

Molecular vaccines for malaria

Joseph T. Bruder,^{1,*} Evelina Angov,^{2,3} Keith J. Limbach^{2,4} and Thomas L. Richie^{2,4}

¹GenVec, Inc., Gaithersburg, MD USA; ²US Military Malaria Vaccine Program; ³Walter Reed Army Institute of Research; ⁴Naval Medical Research Center, Silver Spring, MD USA

Key words: malaria, vaccine, antigen, adjuvant, vaccine design, vaccine delivery, vaccine safety

Abbreviations: Ad5, adenovirus serotype 5; CSP, circumsporozoite protein; TRAP, thrombospondin-related adhesion protein; SSP2, sporozoite surface protein-2; LSA1, liver stage antigen-1; LSA3, liver stage antigen-3; AMA1, apical membrane antigen-1; MSP 1, merozoite surface protein-1; MSA 2, merozoite surface antigen 2; EMP1, erythrocyte membrane protein-1; EXP1, exported protein-1; Pfs25, surface protein-25; *P*, plasmodium; *Pf*, *Plasmodium falciparum*; *Py*, *Plasmodium yoelii*; *Pk*, *Plasmodium knowlesi*; *Pv*, *Plasmodium vivax*; *Pb*, *Plasmodium berghei*; APCs, antigen presenting cells; DCs, dendritic cells; GIA, growth inhibition assays; GPI, glycosylphosphatidylinositol; IFN γ , interferon gamma; TLRs, toll-like receptors; VLPs, virus-like particles; SAPN, self-assembling polypeptide nanoparticle; FMP1, *P. falciparum* MSP1₄₂ 3D7 malaria subunit vaccine; FMP2.1, *P. falciparum* AMA1 3D7 malaria subunit vaccine; GAS, genetically attenuated sporozoites; RAS, radiation-attenuated sporozoites; LSA-NRC, *P. falciparum* liver stage antigen 1 3D7 malaria subunit vaccine; ME, multi-epitope string; RTS,S, CSP-based malaria subunit vaccine

The basic premise of vaccination is the triggering of host immune responses leading to the induction of adaptive immunity having sufficient magnitude and duration to provide long term protection. This has been achieved by many licensed vaccines, the majority based on attenuated or inactivated organisms, although often the protective antigens and underlying molecular mechanisms have not been identified. However, this traditional approach has not led to the development of a licensed vaccine for malaria or for several other devastating infectious diseases. Recently, substantial efforts have been focused on applying rational molecular design principles toward the development of novel vaccines for these refractory pathogens. In this review, we discuss the molecular aspects of antigen design, adjuvant advancement and the development of vaccine delivery systems as they are being applied to malaria vaccines.

Introduction

Molecular vaccines, as defined in this review, are vaccines that are rationally designed at the molecular level to induce long term immunity, optimally comprising both antibody and T cell-mediated responses specifically targeting one or more protective antigens. Molecular vaccines are to be contrasted with whole organism vaccines, either attenuated or inactivated, for which underlying molecular components have not been characterized. While attenuated whole organism vaccines constitute the majority of vaccines currently licensed for human use, they can be viewed as occupying one end of a spectrum of approaches to vaccine development, namely the empirical selection and testing of an immunogen

without specific knowledge regarding the important constituent antigens or the protective immune responses the antigens induce.[†]

At the other end of the spectrum lie rationally designed molecular (subunit) vaccines. Ideally, these are based on (1) understanding pathogenesis at the molecular level, (2) conceptualization of how pathogenic processes could be interrupted (e.g., blocking binding interactions mediating host cell entry, cytoadherence, nutrient uptake, etc.), (3) identification of specific target molecules based on this concept (e.g., the parasite ligand binding to the host receptor), (4) identification of an immune response able to target the key parasite molecules and interrupt the pathogenic process, and (5) development of an appropriate vaccine delivery system, immunogen(s) and formulation capable of inducing the required, antigen-specific, protective immune responses. Figure 1 compares these two divergent approaches.

While we have few examples of licensed molecular vaccines, the approach is appealing, especially for potentially lethal pathogens such as HIV where the development of attenuated whole organism vaccines entails daunting safety concerns, and for pathogens where the development of attenuated vaccines is limited by the difficulty with in vitro production such as malaria. Parasites fall primarily into the latter category—they are difficult to culture and in addition, crude preparations of parasite antigens, such as killed parasites or parasite lysates have proven minimally protective, probably because the natural infection itself often fails to induce sterilizing immunity. This lack of naturally-induced sterile protection reflects the many adaptations by parasites enabling them to circumvent the host immune response, thereby permitting establishment of the chronic infections required to achieve transmission.¹

Malaria represents a poster child for the application of molecular design, due to its supreme public health importance, the

*Correspondence to: Joseph T. Bruder; Email: jbruder@genvec.com
Submitted: 10/20/09; Accepted: 10/29/09
Previously published online:
www.landesbioscience.com/journals/vaccines/article/10463

[†] Although neutralizing antibodies are often a correlate of the protection afforded by whole organism vaccines, this does not mean that they constitute the only protective response; cell-mediated immunity may contribute to protection in many cases.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE OCT 2009		2. REPORT TYPE		3. DATES COVERED 00-00-2009 to 00-00-2009	
4. TITLE AND SUBTITLE Molecular vaccines for malaria				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Medical Research Center,US Military Malaria Vaccine Program,503 Robert Grant Avenue,Silver Spring,MD,20910				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Same as Report (SAR)	18. NUMBER OF PAGES 24	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

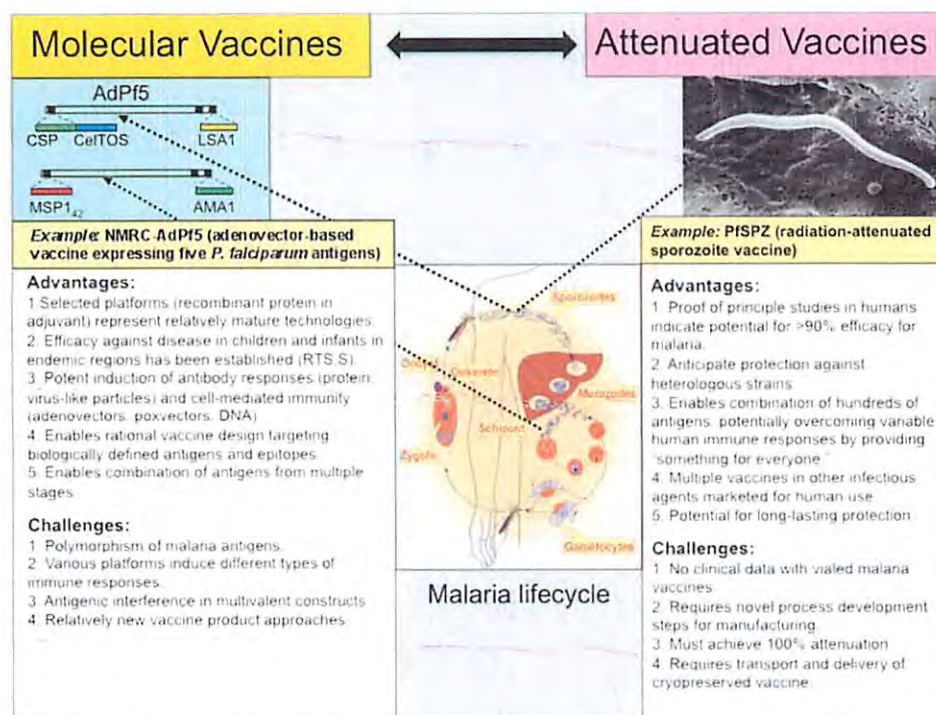


Figure 1. Spectrum of vaccine design. Reproduced in modified form and with permission from Thomson Reuters and Epstein JE, Giersing G, Mullen G, Moorthy V, Richie TL. Malaria Vaccines: Are We Getting Closer? *Curr Opin Mol Ther* 2007; 9:12-14. Copyright 2007, Thomson Reuters (Scientific) Ltd.

presence of multiple parasite-host molecular interfaces (allowing the conceptualization of many opportunities for molecular-based intervention), good animal models and the ability to safely challenge humans with infectious sporozoites to look for protection, which strongly facilitates the iterative testing and refinement of molecular-based vaccine concepts. Even with a molecular approach, however, malaria's complex life cycle and immune invasion adaptations pose extraordinary challenges for the vaccine developer.

Many of the vaccines discussed in this issue of *Human Vaccines* are molecular in nature, including pregnancy-specific vaccines based on interruption of binding to placental chondroitin sulfate A (reviewed by Menendez et al., 21), transmission-blocking vaccines aimed at inducing antibody responses to sexual stage surface antigens (discussed in reviews by Robert Sinden, p. 3 and Birkett p. 139), asexual stage vaccines aimed at inducing antibody responses to variable antigens expressed on the surface of infected erythrocytes (reviewed by Hviid p. 84) and recombinant protein-based vaccines composed of immunodominant proteins, sometimes with well-described functions (reviewed by Anders et al. p. 39). RTS,S (reviewed by Cohen et al., p. 90) is a particularly elegant example of molecular design applied to a recombinant protein, combining rational antigen choice (the vaccine uses a major surface protein of the sporozoite, called the circumsporozoite protein, CSP) with construction of a fusion gene incorporating the target antigen linked to hepatitis B surface antigen, which facilitates self-assembly of the yeast-produced protein into highly immunogenic particles.[‡]

Importantly, RTS,S has shown protection in several Phase 2 field trials and thereby demonstrates the feasibility of developing a molecular vaccine that protects against malaria. Other vaccines reviewed in this issue, such as whole sporozoites genetically attenuated by targeted gene deletion (reviewed by Vaughan et al., p. 107), although based on empiric strategies, incorporate molecular techniques and thus could be considered molecular in design.

To avoid duplication with these accounts of molecular vaccines and molecular approaches, this article focuses on molecular technologies not discussed in detail elsewhere, with an emphasis on gene-based vaccine platforms, such as DNA and viral vectors. It is organized into six sections, the first five emphasizing major vaccine components where molecular design can be applied: (1) antigen design, (2) adjuvant design, (3) vaccine delivery systems, (4) virus-like particles and (5) safety. The review concludes with a discussion of specific challenges in molecular vaccine development for a parasite that has evolved over millions of years to persist in human populations (6).

Antigen Design

Molecular vaccines require specific antigen design considerations, relative to inactivated or attenuated whole organism based

[‡] The mechanism of protection afforded by RTS,S is not well understood; in addition, the vaccine depends upon an adjuvant system that is only partially characterized at the molecular level.

vaccines. For example, both recombinant protein- and gene-based vaccines are candidates for codon optimization or harmonization^{††} to enable improved expression in the relevant production cell line or in humans, and may benefit from additional customized alterations for increasing antibody or T cell responses. Several of the most important aspects to antigen design are discussed in this section.

Molecular modification of antigen expression, localization, secondary processing and intrinsic antigenic properties to improve immunogenicity. The low GC content of the *P. falciparum* genome (~20% relative to ~40% for humans) is associated with different patterns of codon utilization compared to the genes of humans. These different codon usage patterns can negatively impact transgene expression and immunogenicity associated with DNA and viral vectors expressing *P. falciparum* genes in vivo. One of the simplest, most effective ways to increase protein expression is to synthesize a new gene encoding the same amino acid sequence, but with codons that are preferentially used by the species receiving the vaccine (e.g., human, mouse, monkey). Codon optimization has been shown to enhance protein expression of HIV1 and malaria genes encoded by DNA vectors by 4–1,000 fold and to improve the immunogenicity of these vectors in mice.^{3,4} As a result of these findings, codon optimization has become a standard practice for enhancing the expression and immunogenicity of DNA and viral vaccine vectors. However, recent data indicate that codon optimization may not always provide benefit, particularly for cell-mediated immunity. In a study of DNA vaccines encoding two *P. yoelii* antigens and one *P. falciparum* antigen, antibody responses were enhanced for two of the three constructs while cell-mediated responses, as measured by interferon-gamma (IFN γ) ELISpot, lymphoproliferation and cytotoxic T lymphocyte assays were stronger with native sequences. Protection against *P. yoelii* sporozoite challenge was also not improved by codon optimization.⁵ Thus, the decision whether or not to codon optimize is best made in the context of immunogenicity studies, with knowledge of the immune response required for protection.

A second key feature for gene-based vaccines is targeting genes for intracellular vs. cell surface expression. Secretion or cell surface expression as opposed to intracellular expression of antigens can induce a more robust antibody response in the context of vaccine delivery, as extracellular or surface localization leads to antigen presentation via the exogenous antigen presentation pathway. Many *P. falciparum* proteins, including apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1) and CSP contain functional signal sequences and it may be critical to include these in antigens that are to be delivered by gene-based technologies when antibody responses are the priority, whereas these sequences are often deleted in subunit, protein-

based vaccines. Our results indicate that adenovector-mediated expression of AMA1 and MSP1₄₂ (42 kilodalton fragment of MSP1) at the cell surface is associated with improved magnitude and functionality of antibody responses relative to intracellular expression.⁶ This finding is in agreement with other published data for DNA and poxvirus vectored vaccines. In contrast, induction of AMA1 and MSP1₄₂-specific T cell responses following adenovector delivery was not affected by subcellular localization of antigen.

Another factor that could significantly influence immunogenicity is the presence or absence of glycosylation sites. *P. falciparum* parasites do not contain significant amounts of N-linked and O-linked carbohydrates.⁷ However, when produced in, or delivered to eukaryotic cells with viral- or plasmid-based delivery systems, many *P. falciparum* proteins, such as AMA1 and MSP1, are expected to be glycosylated. Non-native glycosylation of these antigens could obscure or alter key epitopes derived from the tertiary structure of these blood stage antigens, and induce antibody responses that are inferior to responses that would be observed with non-glycosylated antigens. Consistent with this, it has been reported that a non-glycosylated *P. falciparum* MSP1₄₂ subunit protein produced in goat milk was a more effective vaccine candidate than the corresponding native sequence glycosylated version.⁸ However, other studies with MSP1₄₂, AMA1 and erythrocyte binding antigen-175 (EBA175) subunit protein vaccines and DNA-AMA1 and DNA-MSP1₄₂ vectors have indicated that glycosylated proteins can be effective vaccines.^{8–11} We have recently shown that adenovectors that express glycosylated versions of MSP1₄₂ and AMA1 induced potent functional antibody responses by an in vitro growth inhibition assay (GIA), and that modification of the N-linked glycosylation sites did not improve and in some cases significantly reduced antibody responses.⁶ Mutation of glycosylation sites may have destroyed immunogenic epitopes directly or may have had negative effects on protein folding and structure.

The CSP protein contains a glycosylphosphatidylinositol (GPI) anchor, which functions to attach the protein to the plasma membrane of the sporozoite. The immunogenicity of DNA or adenovirus vectors expressing *P. berghei*, *P. yoelii* and *P. falciparum* CSP can be improved by deletion of the GPI anchor sequence.^{12,13} We have demonstrated that an 11 amino acid C-terminal deletion, that removes the GPI anchor sequence, increases PfCSP-specific antibody responses in mice and rabbits following adenovector delivery (JTB, unpublished data).

A poorly understood feature of malaria proteins is the degree to which they have been subject to changes in amino acid sequence in response to immunological selection pressures. Epitopes found within proteins that are functionally important to parasite biology (such as ligands mediating host cell invasion) may have evolved, via changes in amino acid sequence, to minimize the induction of protective immunity. One way this could have happened would have been evolution of reduced affinity for the binding grooves of common major histocompatibility complex (MHC) class I and class II alleles. Another would have been the development of configurations that stimulate regulatory T cells, thereby quelling rather than inducing immunity. Still another predicted response

^{††} Optimization here refers to the construction of synthetic genes by selecting for each amino acid the most commonly used codon (i.e., the most commonly used codon by the expressing cell substrate), whereas harmonization refers to tailored selection of codons corresponding in frequency of use to the original codon sequence. Harmonization may be necessary for expression in *E. coli* in order to slow down translation at key points so as to permit critical folding events within the ribosomal tunnel.²

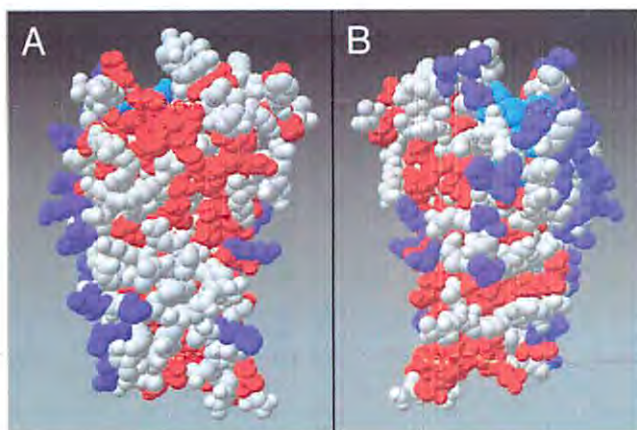


Figure 2. Crystal Structure and Sequence Variability of AMA1, front (A) and rear (B) views of PfAMA1. Residues that are conserved among all *Plasmodium* species for which sequence is currently available are shown in red. These, along with residues that vary between *Plasmodium* species (gray), form a core structure that underlies residues that are polymorphic in *P. falciparum* (purple). The polymorphic residues are located exclusively at the molecular surface and show pronounced clustering to the rear face. The putative hydrophobic groove is shown in blue at the top. Original figure by Bart Faber and Henk van Westbroek, using model 1Z40 from the Protein Data Bank (www.pdb.org) provided by Bai et al.²³⁸ The Swiss pdb viewer (<http://www.expasy.org/spdbv/>) was used to generate the figure.²³⁹

would be the evolution of highly immunodominant epitopes in other domains of the protein, exemplified by the repeat sequences characterizing many malarial proteins. These immunogenic domains could serve to divert the immune response away from the critical functional epitopes, leaving them “immunologically silent.”

Although these various predicted features have not been systematically described for most malaria antigens, there is a growing list of examples concordant with these principles. One is the existence of cryptic epitopes—normally immunologically silent epitopes that when removed from the protein microenvironment can induce protective responses.^{14–17} Another is altered peptide ligands (T cell mimotopes)—parasite-derived domains that appear to induce inhibitory rather than protective immune responses.^{18–20} These antagonist peptides, which may differ in one or two amino acids from the agonistic peptide, are hypothesized to decrease the threshold of T cell activation, induce T cell anergy, or alter the CD4 T cell phenotype from pro-inflammatory (Th1) to anti-inflammatory (Th2).¹ A particularly interesting example of the interplay of immune selection and epitope sequence and structure is provided by AMA1, described in Figure 2. In this case, highly polymorphic residues surround an invariant (and possibly immunologically silent) putative binding site.

Strategies for reversing these parasite adaptations have been explored, but much further work is needed. For example, targeted amino acid substitutions may restore the ability of key epitopes to bind with higher affinity to MHC molecules, or prevent the induction of regulatory T cells. While changing the amino acid structure of a protein might induce immune responses less

specific for the native protein, it may be possible to make limited substitutions that enhance rather than reduce the affinity of antibodies or T cells for the native protein (T. Husain, personal communication). Efforts to do this in malaria and other disease models have yielded mixed results,^{21, 22} but given the often poor immunogenicity of malaria epitopes, the concept merits further investigation. Additional methods to focus immune responses on protective epitopes are discussed in the next section, in the context of overcoming the challenges provided by antigenic polymorphism.

Molecular approaches to circumventing antigenic polymorphism. The malaria parasite is notable for the antigenic polymorphism exhibited by many of the proteins that have been developed as vaccine candidates. Perhaps the best example is the *P. falciparum* erythrocyte membrane protein-1 (PfEMP1), an immunodominant adhesion protein mediating cytoadherence of parasitized erythrocytes to the vascular endothelium. PfEMP1 is expressed from approximately 60 different paralogous genes encoding immunologically variant versions of the antigen (*var* gene family). These variants are expressed, in a sequentially regulated manner, on the surface of protuberances that appear on the membrane of infected erythrocytes during the late trophozoite stages. This evasion strategy enables the escape of new variants from neutralization by the antibody responses specific for preceding variants, resulting in recurrent waves of parasitemia. Orthologs of the *var* and similar variant gene families, such as *rif*, *stevor* and *Pfmc-2TM* are found in many malaria species and appear to be a common adaptation resulting from immune-mediated selection.^{23, 24} Strategies for designing vaccines to overcome the polymorphisms presented by variant gene families are discussed in the review by Lars Hviid.

Individual genetic loci also exhibit striking allelic variation, typified by AMA1, the blood stage antigen mentioned above. AMA1 is a promising target antigen for blood stage malaria vaccine development, as demonstrated in animal challenge experiments and in epidemiological studies of humans living in endemic areas. The main issue for vaccine developers is that hundreds of variant alleles of AMA1 have been described (Box 1) and antibodies specific for one allele do not efficiently cross-neutralize other strains.²⁵ Such polymorphism indicates both the potential importance of variant antigens as vaccine candidates (if they were not protective, they would not have been subject to immune selection) as well as the difficulty faced by vaccine developers in constructing a fully cross-protective vaccine.

The Laboratory of Malaria Immunology and Vaccinology (LMIV, formerly the Malaria Vaccine Development Branch) at the National Institutes of Allergy and Infectious Diseases and the Walter Reed Army Institute of Research (WRAIR) have each developed recombinant protein vaccines based on AMA1 and these have been tested by the Malaria Research Training Center (MRTC) in Mali for efficacy against clinical infections in children. The former vaccine did not protect,²⁶ likely as a result of the extensive allelic diversity present at the study site, and the results of the latter trial are not yet publically available. It will be important in such trials to undertake allelic shift (sieve) analyses, to see if incident infections in the vaccine group demonstrate a paucity

Box 1. AMA1 – A Study in Polymorphism. AMA1 is an 83kDa type I integral membrane protein relatively conserved among apicomplexan protozoa. In *Plasmodium* species, AMA1 is maximally expressed during the formation of merozoites in late schizogony, targeted to the microneme apical organelles and secreted onto the surface of the merozoite at the time of invasion of the host erythrocyte. AMA1-based vaccines provide protection against malaria challenge in animal models (reviewed by Remarque),²⁴⁰ induce antibodies which inhibit the growth of *P. falciparum* in culture²⁴¹ and correlate with protection against clinical malaria in endemic areas.²⁴²⁻²⁴⁴ AMA1 is also expressed in the sporozoite and liver stages of the parasite,²⁴⁵ raising the possibility that immune responses to this antigen could simultaneously protect against multiple stages of the parasite life cycle. Although the exact role of AMA1 in malaria biology has not been elucidated, cleavage steps are integral to its release onto the surface of the merozoite²⁴⁶ and to subsequent erythrocyte invasion.^{247,248} Parasites lacking the AMA1 gene, which occurs as a single copy in the genome, are not viable.²⁴⁹

The major portion of the molecule, the ectodomain, possesses a remarkable crystal structure characterized by a conserved central core and variable external loops formed by three different interlocking subdomains (Fig. 2).²⁵⁰ A hydrophobic groove on the face is surrounded by polymorphic residues,²³⁸ which may signify epitopes serving to divert the immune response from cryptic epitopes formed by the potential binding site lying within the groove.²⁷

AMA1 illustrates the barrier facing the development of molecular vaccines posed by antigenic polymorphism. A review of AMA1 identified 64 polymorphic residues based on sequences listed in GenBank, with only three of the 64 in the conserved C-terminal cytosolic region, nine in the N-terminal pro-sequence and the large majority, 52, in the ectodomain.²⁵¹ This diversity likely represents only the tip of the iceberg, as illustrated by studies conducted over three years in Mali revealing equivalent diversity at a single vaccine testing site, including 62 polymorphic amino acid positions (46 dimorphic, 13 trimorphic, 3 tetra-hexamorphic) most of which were clustered on the surface of the ectodomain with the greatest concentration around the edges of the hydrophobic groove. The 506 single strains identified in the 748 infections assessed at a study site in Mali demonstrated 214 unique parasite AMA1 haplotypes.²⁸

These estimates of diversity pose an important question for the molecular vaccine enterprise: how can this remarkable polymorphism be reconciled with a subunit vaccine approach?

of the vaccine or other cross-reactive haplotypes relative to those in the control group. Demonstrating allele-specific protection will provide a foundation for building a more broadly protective vaccine by enumerating the number of alleles required.

Dutta et al. at the WRAIR have explored strategies to develop a more broadly protective vaccine using an in vitro GIA and mutant AMA1 proteins. They identified specific polymorphic residues that function as antigenic escape residues (AERs), meaning that polymorphisms at these sites escape cross-protection in the GIA.²⁷ The primary AERs map to a cluster of polymorphic residues on domain-1 (C1 cluster) that induce strain-specific antibodies. Narrowing down the AMA1 polymorphic sites to a small number of critical AERs is an important step in the molecular design of an AMA1 immunogen(s), because it will facilitate allelic shift analyses during Phase 2b trials.

As a complementary approach, the Malaria Research and Training Centre (MRTC) in Mali in a collaboration with the University of Maryland, examined the probability that the second of two sequential infections experienced by participants in a longitudinal clinical study is symptomatic, based on the degree

to which the AMA1 molecule sequenced from the second infection differed from that of the initial infection. This analysis has identified a relationship between the degree of genetic distance between the AMA1 alleles from the first and second infection and the likelihood of clinical illness, with amino acid substitutions within the AERs appearing to be particularly important.²⁸ If sufficient data are collected from study sites across the world, this approach should enable down-selection of naturally occurring haplotypes maximizing the degree of cross-protection.

Other vaccine developers are also using GIA to help design a broadly protective AMA1 vaccine. The LMIV at NIH in collaboration with MRTC in Mali has applied a clustering algorithm to AMA1 sequence data from isolates around the world and identified six distinct populations based primarily on geographic area that in limited testing have induced non-cross-protective antibodies, thus constituting a minimum requirement for the number variants needed for a vaccine.²⁹ The Biomedical Primate Research Center (BPRC) in The Netherlands has synthesized three “diversity covering” versions of AMA1 encompassing 97% of the amino acid variability observed in nature and conducted preliminary studies in rabbits showing the induction of cross-inhibitory antibodies.³⁰ Similar studies are underway at the La Trobe University and at the Walter and Eliza Hall Institute of Medical Research in Australia.

A radically different strategy is to avoid full length vaccine antigens with their variant sequences, focusing instead on the identification of structurally constrained, conserved epitopes associated with key parasite functions. The conserved, hydrophobic trough of AMA1 may play such a critical role, binding to other molecules involved in merozoite invasion (Fig. 2). The fact that monoclonal antibodies that inhibit invasion in vitro appear to act by hindering this binding interaction^{31, 32} may indicate that protective immunity could be induced by a vaccine focusing immune responses on a single or small number of epitopes.

Several approaches can be taken to targeting individual conserved epitopes. One is to “cloak” potentially diversionary epitopes (such as those surrounding the hydrophobic trough of AMA1) by coating them with non-immunogenic molecules, allowing the immune system to focus on those conserved epitopes most likely to be protective.³³ Another is to synthesize epitope strings—linked sequences of conserved epitopes designed to bind to an array of MHC molecules, allowing broad population coverage for the vaccine, selecting only the epitopes of interest.³⁴ These epitope strings can be designed to include complementary sets of variant epitopes from polymorphic antigens, as well as conserved epitopes. Epitope strings, however, do not provide conformational structure, so may not be suitable for inducing antibodies to a putative binding site. A third approach for addressing antigen diversity is represented by “gene shuffling”, whereby a series of variant alleles are broken into short segments and then various hybrid alleles are randomly re-assembled. Multiple shuffled products are evaluated via an immunoscreening assay to down-select those inducing the most broadly neutralizing antibodies. Gene shuffling has been applied to malaria vaccines with promising results in pre-clinical studies.³⁵

These approaches illustrate the efficiency and versatility of molecular design for malaria vaccine development when applied to overcoming antigenic polymorphism. The techniques potentially (1) focus immune responses on conserved, protective epitopes by eliminating undesired antigens or domains, (2) provide a strategy for inducing, from one vaccine construct, a broadly neutralizing immune response targeting a highly polymorphic antigen, and in the case of epitope strings (3) offer a strategy for combining different antigens from the same or different stages of the malaria parasite life cycle. Antigen combination is further discussed in the next section.

Antigen combination strategies. The feasibility of a highly effective malaria vaccine is strongly supported by the robust (>90%) sterile immunity induced in humans by immunization with the radiation-attenuated sporozoite (RAS) vaccine^{36,37} (see review by Stephen Hoffman). This protective immunity appears to target multiple antigens, as evidenced by the identification of genetically restricted responses to pre-erythrocytic (sporozoite and liver) stage antigens other than CSP in the RAS vaccine-immunized volunteers,^{34, 38-40} and by the fact that 100% protection can be induced by the RAS vaccine in tolerized mice unable to mount any immune response to CSP.⁴¹

Additional support for the concept of a multi-antigen vaccine comes from the fact that malaria vaccine-candidates based on single antigens have been poorly efficacious, with the exception of the RTS,S vaccine. Based on the high level of protection afforded by RAS and by the closely related approach of gene-knockout attenuation (genetically attenuated sporozoites, or GAS), a highly efficacious malaria vaccine will likely need to target multiple antigens. It is well documented that genetic restriction of host immune responses, sequence polymorphism of critical epitopes targeted by T cells and B cells, allelic variation and antigenic diversity pose enormous obstacles for the development of a malaria vaccine based on only a single antigen.⁴² These challenges may be overcome by the inclusion of multiple target antigens into a vaccine.

Doolan et al.⁴³ showed that inclusion of two antigens in a *P. yoelii* DNA vaccine enhanced protection on multiple genetic backgrounds relative to DNA vaccines based on each of the individual plasmid components. This work suggests that in a heterogeneous human population inclusion of multiple antigens in a malaria vaccine may circumvent the genetic restriction imposed by the host genetic background and improve vaccine efficacy in the population.

Data from the *P. knowlesi* (*Pk*) non-human primate challenge model further supports the contention that candidate malaria vaccines should include multiple antigens.⁴⁴⁻⁴⁶ Immunization of rhesus monkeys with a DNA prime/poxvirus boost regimen was partially protective only when the DNA and poxviruses expressed four antigens [*Pk*CSP, *Pk* thrombospondin-related adhesive protein/sporozoite surface protein-2 (*Pk*TRAP/SSP2), *Pk*AMA1 and *Pk*MSP1]; substantial delays were observed to >2% parasitemia in most animals and 2/5 animals self-cured their parasitemia. In contrast, regimens where both vectors expressed only one antigen (*Pk*CSP) provided only minor delays to >2% parasitemia and no self-cures.⁴⁴ The protection afforded by this four-antigen,

multi-stage vaccine was confirmed in a second study where the interval between the DNA prime and the poxvector boost was extended to 44 weeks; in this case 3/5 monkeys were sterilely protected and the remaining two animals show a delay to the first day of parasitemia.⁴⁵ Based on these results it would be of interest to develop and test a similar multi-antigen, multi-stage vaccine in humans.

One of the critical factors associated with the combination of antigens is assuring that the selected multi-antigen mixture is internally compatible. Elegant studies immunizing mice with plasmid "cocktails" by Sedegah et al. demonstrated that plasmid mixtures inhibited the immunogenicity of individual plasmids, and that particular plasmids may have been responsible for much of this inhibition. Removing inhibitory plasmids from the cocktail restored the immunogenicity of the remaining plasmids, which then induced antibody and T cell responses equivalent or nearly equivalent to those induced when the antigens were administered by themselves.⁴⁷ Transfection studies further indicated that the mixtures may have reduced RNA transcript levels and that this may have been related to competition among the CMV IE promoters used to drive transgene expression.⁴⁸

Another way to combine antigens while potentially avoiding antigenic competition is epitope strings, as discussed in the preceding section. Two such poly-epitope strings representing multiple antigens have been tested in the clinic. The first, consisting of epitopes from several different pre-erythrocytic stage *P. falciparum* proteins, was designed for co-expression with full length TRAP/SSP2. The combined antigen (multi-epitope-TRAP or ME-TRAP), has been delivered via DNA, pox and adenovectors and significantly reduces liver stage burden in humans following *P. falciparum* sporozoite challenge, showing delays in the onset of parasitemia and sometimes sterile protection when delivered in prime-boost combination, these effects attributed primarily to the TRAP antigen but with the epitope string possibly contributing (see review by Adrian Hill). The Oxford group has also tested a similar polyprotein comprising longer portions of six pre-erythrocytic stage antigens, but without linkage to TRAP/SSP2. Although the latter vaccine was safe and moderately immunogenic in humans,⁴⁹ there was no protection against experimental sporozoite challenge (A. Hill, personal communication). Two other polypeptide vaccines are nearing clinical testing. The first is a protein-based construct called FALVAC-1A, being developed by the Center for Disease Control and Prevention.⁵⁰ The second, originally developed by the US Navy and Epimmune, Inc, is DNA-based and is now being developed by NIAID for clinical testing, currently scheduled for 2010 (A. Mo, personal communication).

Adjuvant Design

The spectrum of vaccine design discussed in the introduction, with live-attenuated vaccines at one end and rationally designed subunit vaccines at the other simultaneously defines a spectrum in the requirement for adjuvants. Replicating vaccines generally do not require adjuvants to stimulate potent immune responses, while recombinant protein and peptide-based vaccines invariably

do. DNA, viral vectors and virus-like particles possess inherent properties for activating the immune system and traditionally have not required adjuvants. However, it is now clear that their potency may be improved with novel molecular designs, including co-formulation with a variety of immunostimulatory molecules. These and other developments are discussed in this section, beginning with traditional adjuvants and progressing to novel molecular adjuvants for protein-, peptide- and gene-based vaccines, focusing on molecular design.

Adjuvants can be represented by several classes of compounds, such as microbial products, mineral salts, emulsions, microparticles, and liposomes, which can exert their function by various, still poorly characterized mechanisms of action. Some of the modes of action attributed to adjuvants in potentiating immunity, whether innate or adaptive, include antigen uptake by antigen presenting cells, upregulation of antigen presentation and localization to the draining lymph node, increased cellular distress and the antigen depot effect. After decades of intensive investigations, recent advances led to better understanding of the mechanism of action of immunomodulatory compounds as they relate to induction of innate immunity, opening the doorway for improving adjuvant design at the molecular level.

Adjuvants for Proteins and Peptides. Only a handful of adjuvant formulations are currently approved for human use. In the USA, aluminum salts (aluminum hydroxide or aluminum phosphate, generically referred to as alum) are the only example of an FDA approved adjuvant. Alum has been used extensively over several decades in vaccine formulations for diphtheria, tetanus, pertussis, hepatitis A, hepatitis B and other infectious agents. Although able to efficiently enhance humoral immune responses, alum is relatively ineffective for inducing cell-mediated immune responses.

In the European Union (EU), MF59, a squalene-based oil-in-water emulsion, was recently licensed for a flu vaccine formulation (Fluad), representing a second adjuvant approved for human use. GlaxoSmithKline Biologicals (GSK) is also testing novel adjuvants, developing numbered "Adjuvant System" (AS) technologies that are based on combinations of classical adjuvants, such as aluminum salts, oil-in-water (o/w) emulsions, liposomes and immunomodulatory molecules (reviewed in Garçon et al.).⁵¹ The objective is to induce tailored immune responses directed against the pathogen. An example is provided by AS02, which has been tested in several malaria subunit vaccines, including RTS,S, FMP1, FMP2.1 and LSA-NRC.⁵² AS02 is an o/w emulsion containing MPL (3-O-desacyl-4'-monophosphoryl lipid A), an immunostimulatory molecule derived from the detoxified cell wall of Gram-negative *Salmonella minnesota* R595 absorbed to alum, and QS21 (saponin), derived from Quil A, a fractionate from the bark of *Quillaja saponaria*, a Chilean tree. AS02 has induced high antibody titers and strong cell-mediated (primarily CD4⁺ T cell-mediated) immune responses characterized by high levels of interferon-gamma (IFN γ) secretion. Generally, the immunostimulant MPL has been shown to induce the synthesis and release of interleukin-2 (IL-2) and IFN γ , promoting the generation of Th1 responses,⁵³ while QS21 (saponins) functions mainly through the induction of other cytokines.

In an effort to further strengthen cellular responses, particularly CD8⁺ T cell responses, GSK has developed a new formulation, composed of liposomes, MPL and QS21, called AS01. In an adjuvant comparison study in rhesus macaques, RTS,S/AS01 elicited higher RTS,S-specific antibody titers and higher numbers of antigen-specific IFN γ producing cells than RTS,S/AS02.⁵⁴ Following from these encouraging results, RTS,S/AS01 advanced into Phase 1/2b efficacy trials in pediatric populations and is now transitioning into Phase 3 pre-licensure trials. Another adjuvant formulation in the GSK repertoire, AS04, is a Toll-like receptor (TLR) 4 agonist comprised of MPL in alum. AS04 was recently approved for human use in the EU for hepatitis B virus (Fendrix) and human papilloma virus (Cervarix) vaccines. More recently, AS03, an oil-in-water emulsion, was approved as a component of a pre-pandemic H5N1 vaccine (Prepandrix).

Montanides are biodegradable emulsions containing squalene and mannide-monooleate as emulsifier and have similar physical characteristics as incomplete Freund's adjuvant. Some promising results have been reported in previous malaria vaccine trials^{55, 56} using Montanide ISA 720 (SEPPIC, Paris, France), possibly due to the slow-release capacity of the inert water-in-oil emulsion and immune stimulating effects of its components. Although very potent immunologically, results from more recent malaria trials using montanides (ISA 51 and ISA 720) have led to the conclusion that this class of adjuvant may have limited potential for further development due to local reactogenicity, including the induction of sterile abscesses and erythema multiforme.^{57, 58}

Even as these various peptide/protein adjuvants are actively developed, understanding of the molecular mechanisms underlying their immunopotentiating properties remains modest. Development is driven empirically by the safety, immunogenicity and protection identified in pre-clinical and clinical testing, with purposeful molecular design playing a less central role. Several new classes of adjuvants, however, are closely linked to defined molecular pathways. These are best exemplified by TLR agonists, discussed in the next section.

TLR agonists. TLRs are pattern recognition receptors found on different cell types that recognize specific molecular patterns on pathogens like viruses, bacteria, and fungi.⁵⁹⁻⁶¹ TLR agonists act as potent adjuvants by activating key antigen presenting cells (APCs), such as dendritic cells (DCs), through their cognate TLRs. These TLR-dependent pathways up-regulate cytokine secretion, increase the expression of MHC class II and co-stimulatory molecules, and promote DC migration to the T cell area of the lymph node.⁶² A variety of immunostimulatory TLR agonists targeting specific TLRs are currently being investigated for use as adjuvants in vaccine formulations; for example heat shock proteins (TLR 4), flagellin (TLR 5), porins (TLR 2), LPS (TLR 4), triacylated and diacylated lipopeptides (TLR 1/TLR2 and TLR 2/TLR 6, respectively) and oligodeoxynucleotides (ODNs) such as unmethylated deoxycytosine deoxyguanosine (CpGs) (TLR 9) (reviewed in 63).

From the molecular design perspective, TLR agonist delivery can be achieved through direct conjugation to an antigen or through incorporation into targeting vehicles, such as viral particles, liposomes, synthetic microparticles, particulates or

bacterial vector platforms. For example, the 19kDa C-terminal fragment of *P. vivax* MSP1 (PvMSP1₁₉) was co-administered with the *Salmonella enterica* serovar typhimurium flagellin (FliC), a TLR 5 agonist, to C57BL/6 mice by either parenteral (s.c.) or mucosal (i.n.) routes. FliC was either mixed or genetically linked to the PvMSP1₁₉ protein. This approach elicited strong, long-lasting MSP1₁₉-specific antibody responses that were biased toward IgG1 subclass. Inclusion of CpG, a TLR 9 agonist, yielded a shift toward a balanced IgG1/IgG2c ratio and higher cellular responses as measured by IFN γ secretion.⁶⁴ In a recent study, a synthetic, FDA approved topical treatment for skin conditions, imiquimod, a TLR 7 agonist, was shown to function as a potent adjuvant for eliciting T cell responses. Topical application of imiquimod at the site of subcutaneous injection of *P. falciparum* CSP peptides elicited parasite-specific humoral immunity that protected C57BL mice against mosquito-bite challenge using transgenic rodent parasites expressing *P. falciparum* CSP repeats.⁶⁵

ODN CpG motifs are recognized by TLR 9 expressed on phagosomes of specialized cell subsets, such as B cells and plasmacytoid dendritic cells. CpG ODNs have been primarily used to induce polarized Th1 type immune responses.^{66,67} Several studies have shown that antigens and CpG ODN must be co-localized to the same APC to generate the most potent and effective therapeutic antigen-specific immune responses. Delivery vehicles can be used to ensure the co-delivery of antigens and CpG ODN to the same APCs, thus leading to significant increased uptake by APCs. These strategies can result in antigen-specific immune responses that are on the order of 5- to 500-fold greater than for antigen alone. For example, in mice, the co-administration of CpG and recombinant surface protein-25 (Pfs25) led to a 30-fold improvement in antibody titers and avidities compared to rPfs25 plus alum alone.⁶⁸ Results from clinical studies using recombinant *P. falciparum* AMA1-C1/Alhydrogel plus CpG 7909 vaccine showed 14-fold-higher AMA1-specific IgG with significantly increased in vitro growth inhibitory activity against homologous parasites compared to AMA1/Alhydrogel alone.⁶⁹ Thus, TLR 9 agonists are effective immunomodulatory molecules for use as vaccine adjuvants.

Adjuvant properties of DNA and viral capsids. In contrast to protein-based subunit vaccines, viral, bacterial, and DNA vectors generally do not require formulation in adjuvant for induction of T cell and antibody responses. However, the intrinsic "danger signals" provided by these delivery systems can be improved further by molecular engineering. For example, the addition of unmethylated CpG motifs into the sequence of a DNA vector expressing a tumor associated antigen has been shown to enhance the immunogenicity and efficacy of the vector in a murine melanoma model.⁷⁰ Conversely, enzymatic methylation of CpG motifs in a DNA-*P. yoelii* CSP vector decreased the immunogenicity of the vector in mice.⁷¹ CpG activation via a TLR 9-dependent mechanism does not appear to be absolutely essential, however. Multiple signaling pathways may be involved, including two recently discovered pathways utilizing the DNA sensors DNA-dependent activator of interferon regulatory factors (DAI) and absent in melanoma-2 (AIM2).⁷² As more is learned about the

role that these different pathways play in activating the innate immune system, it may be possible to use this information to develop better DNA vaccine vectors.

Adenovirus-based vectors activate the innate immune system through TLRs and cytoplasmic DNA-sensing, inflammasome-dependent mechanisms.⁷³⁻⁷⁷ This results in the induction of cytokines and chemokines, which potentiate antigen-specific T cell and antibody responses. Moreover, purified adenovector serotype 2 (Ad2) hexon protein has been shown to be a potent adjuvant for induction of antigen specific responses.⁷⁸ Other viral vectors, including poxvirus^{79, 80} and semliki forest virus,⁸¹ can enhance antigen-specific antibody and T cell responses to co-delivered proteins, indicating that these viral vectors also provide adjuvant activity. These intrinsic adjuvant properties of DNA and viral vectors facilitate the induction of robust immune responses to vector-delivered antigens, bypassing the need for adjuvant formulation.

Molecular adjuvants for plasmid and viral vectors. Although plasmid and viral delivery systems do not require adjuvants, co-formulation with an adjuvant can improve the antigen-specific immune responses induced by these vectors. For example, formulation of DNA vaccines with CpG oligonucleotides improved the immunogenicity of DNA plasmids encoding malaria antigens in mice and Aotus monkeys.^{82, 83} Co-administration of DNA vaccines with plasmids expressing immunostimulatory molecules has likewise improved the immunogenicity of these vaccines in animal models. However, these initial successes have not necessarily translated into humans. For example, when tested in mice, co-delivery of plasmids expressing PyCSP and granulocyte-macrophage colony-stimulating factor (GM-CSF) resulted in more robust antibody and T cell responses, as well as better protection from *P. yoelii* sporozoite challenge relative to PyCSP expressing plasmid alone.^{84, 85} But, when this combination was evaluated in humans, co-delivery of human GM-CSF and *P. falciparum* antigen expressing plasmids did not improve T cell responses relative to the *P. falciparum* antigen expressing plasmids alone, and none of the regimens were protective.⁸⁶

Intervention in costimulatory and inhibitory pathways can modulate immune responses and represent interesting strategies for increasing vaccine responses. The herpes simplex virus glycoprotein D (gD) can enhance T cell and antibody responses to fused antigens in the context of DNA and adenovector delivery.⁸⁷ gD functions by blocking inhibitory signals between the herpesvirus entry mediator (HVEM) and B- and T-lymphocyte attenuator (BTLA). This pathway has also been exploited to increase tumor-specific T cell responses. Interactions between HVEM and LIGHT (a lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry on T cells) provide costimulatory signals for T cell activation. Adenovector-mediated expression of LIGHT within a tumor can induce tumor-specific T cell responses and eradicate spontaneous metastases.⁸⁸ In a similar way, increased understanding of the signaling pathways that activate the innate immune system has potential for enhancing the immunogenicity of DNA and viral vector based vaccines. Recently, coexpression of plasmids encoding the TLR-signaling molecules, TRAM and

TAK1, were shown to significantly enhance antigen-specific T cell responses in mice following DNA vaccination.⁸⁹

Adjuvants can act through a variety of mechanisms. For example, since DNA plasmids injected into the skin or muscle are taken up by cells inefficiently, adjuvants that enhance the uptake of the plasmid into the cell are being evaluated. Particles that have a diameter of approximately 1 μm can be internalized by APCs, such as macrophages and DCs. Therefore, one way to potentially enhance DNA vaccines is to formulate the vector onto a particle. DNA vaccines formulated onto cationic microparticles composed of poly lactide-co-glycolide (PLG) or nonionic poloxamer (CRL1005) particles combined with a cationic surfactant can be internalized by APCs and have been shown to be more potent than unformulated DNA vaccines in pre-clinical animal models of HIV and CMV.^{90,91} A DNA-CMV vaccine formulated in the CRL1005 adjuvant has also been shown to be safe and moderately immunogenic in a Phase 1 clinical trial.⁹² Formulation of DNA vaccines onto PLG and poloxamer particles, however, does not always enhance potency. For example, formulation on PLG microspheres was tested in the rhesus monkey/*P. knowlesi* sporozoite challenge model, using a multistage malaria vaccine administered as three priming doses of DNA, with or without PLG formulation, followed by a poxvector boost. In this study, PLG formulation of the DNA proved less protective than PBS formulation.⁸⁴ A similar multivalent DNA vaccine encoding *P. falciparum* antigens was assessed for immunogenicity in rhesus monkeys with and without the poloxamer CRL1005 and the poloxamer did not significantly improve antibody responses or cell-mediated immunity, leading to a "no-go" decision for clinical evaluation.⁹³

Other adjuvants for DNA vaccines are also being evaluated. Vaxfectin[®] is a cationic lipid that binds to negatively charged DNA. A Vaxfectin[®]-formulated DNA-*PyCSP* vector has been shown to be more immunogenic and efficacious in the *P. yoelii* mouse model than the unformulated vector.⁹⁴ Similar positive results were obtained in a non-human primate model evaluating the immunogenicity of DNA plasmids expressing malaria antigens that were formulated in Vaxfectin[®].⁹⁵

Adenovectors have been adjuvanted by formulation with standard adjuvants and also by modifying capsid components. Formulation of an adenovirus serotype 35 (Ad35)-based vector expressing *PyCSP* with aluminum phosphate adjuvant [AlPO₄(4)] was shown to increase CSP-specific T cell and antibody responses in mice.⁹⁶ Studies in mice and non-human primates indicate that the immunogenicity of Ad35 vectors can be improved by substitution of the Ad35 fiber with the Ad5 fiber.^{97,98} The Ad5 fiber shaft has been implicated in DC-targeting and induction of adaptive immunity by Ad5 vectors through binding a heparin-like receptor.⁹⁹ These studies suggest that the adjuvant quality of adenovirus capsids may be affected by fiber receptor interactions.

Conjugation of molecular adjuvants to Plasmodium antigens can also enhance immune responses and has the potential to improve malaria vaccines. Fusion of the MSP1₉ to the complement inhibitor C4-binding protein (C4bp) enhanced antibody responses and protection of mice following challenge with *P. yoelii*-infected erythrocytes.¹⁰⁰ This fusion protein also induced

stronger T cell responses, higher titer antibody responses and more robust protection in mice when delivered by an adenovirus vector.¹⁰¹ The mechanism for the adjuvant activity of C4bp is not known, but may involve antigen oligomerization or targeting of antigen to antigen presenting cells. Conjugation of malaria antigens, Pfs25 and AMA1, to nontoxic *Pseudomonas aeruginosa* ExoProtein S (rEPA) was shown to increase antibody responses by up to 1000-fold.¹⁰² Codelivery of the toll-like receptor (TLR) ligands^{103,104} and fusion of antigens to the C3d component of complement^{105,106} have shown promise as molecular adjuvants for infectious disease and cancer and may have applications for malaria vaccines.

Antigen Delivery

A highly effective malaria vaccine will likely induce robust protective antibody and T cell responses to multiple malaria antigens expressed at multiple stages in the parasite's life cycle. As discussed in the antigen design section, sequence polymorphism of critical epitopes targeted by T cells and B cells poses enormous obstacles for the development of a malaria vaccine based on only a single antigen.⁴² These challenges may be overcome by the inclusion of multiple target antigens into a vaccine, making antigen combination a high priority for vaccine developers. Leading-candidate platforms that are capable of inducing responses to multiple antigens are DNA viral and bacterial vectors. Molecular design is an important component for each of these delivery platforms.

Plasmids. In the early 1990s, it was discovered that immunization with a plasmid DNA vector-expressing a flu antigen could protect mice against an influenza challenge.¹⁰⁷ Since then, DNA vaccines have been used successfully in many pre-clinical animal studies, including the demonstration that an unadjuvanted DNA plasmid encoding *P. yoelii* CSP could sterily protect mice against a sporozoite challenge through a CD8⁺ T cell-dependent mechanism.¹⁰⁸ The early success of DNA vaccines in mice, however, has not translated into humans. Clinical evaluation of first-generation DNA vaccines against HIV and hepatitis B virus indicated that DNA vaccines were safe, but not very immunogenic.^{109,110} Similar findings were observed with DNA vaccines against malaria. For example, two Phase 1 studies with a DNA vector expressing a non-codon optimized *PfCSP* gene^{111, 112} and a Phase 1/2a study with 5 vectors expressing *PfCSP*, *PfTRAP/SSP2*, *P. falciparum* exported protein-1 (*PfEXP1*), *P. falciparum* liver stage antigen-1 (*PfLSA1*) or *P. falciparum* liver stage antigen-3 (*PfLSA3*), with or without a DNA vector expressing human GM-CSF,⁸⁶ indicated that DNA vectors could induce malaria-specific CTL or ELISpot responses in about half of the human volunteers, but could not induce detectable malaria-specific antibodies.^{86, 113, 114} Furthermore, the Phase 1/2a trial indicated that three doses of five DNA vaccine vectors expressing different pre-erythrocytic malaria proteins could not protect volunteers against a *P. falciparum* sporozoite challenge. The conclusion from all of these trials was that DNA vaccines were safe, but not very immunogenic or efficacious in humans, even when used in combination.

DNA vaccines have been more successful for veterinary targets. Within the last few years, four plasmid DNA products

have been licensed in the USA, Canada or Australia. A DNA vaccine against West Nile Virus has been developed by Fort Dodge Laboratories for horses. A DNA vaccine against infectious hematopoietic necrosis virus has been developed by Aqua Health, a division of Novartis Animal Health, for salmon.¹¹⁵ A therapeutic DNA vaccine against oral melanoma has been developed by Merial Animal Health for dogs.¹¹⁶ A DNA vector expressing a growth hormone to promote growth and reduce mortality has been developed by VGX Pharmaceuticals for pigs.¹¹⁷ Although the licensing of these veterinary products has bolstered the belief that DNA vaccines may be possible for humans, the clinical experience indicates that the potency of these vaccines will need to be dramatically improved before they can be viable in humans.

DNA vaccines can be delivered by several different systems, including a needle and syringe, a needle-free jet injection device (e.g., Biojector®), a particle mediated needle-free device (e.g., gene gun) or an electroporator. The first DNA-malaria vaccine to be evaluated in humans was injected IM with a needle and syringe.¹¹¹ In subsequent trials, DNA-malaria vaccines were also administered IM or IM/intradermally (ID) with a Biojector®, which can deliver intramuscular, subcutaneous or intradermal injections. A direct comparison of these delivery systems indicated that IM administration with a Biojector® elicited higher cellular immune responses than a needle and syringe or IM/ID administration with a Biojector®.¹¹⁸ DNA vaccines have also been administered by a gene gun, which uses a needle-free device to deliver vaccine coated onto gold beads into the skin. This immunization system, which can deliver the vaccine directly into cells, such as APCs, has been shown to enhance the immunogenicity and efficacy of DNA vaccines.¹¹⁹ A trivalent DNA-flu HA vaccine formulated onto gold beads and administered with a gene gun has also been shown to partially protect volunteers against an influenza challenge in a recent Phase Ib clinical trial.¹²⁰

Another promising way to enhance the potency of DNA vaccines is to deliver these vectors with an electroporator. Electroporation involves the administration of brief electrical pulses immediately after intramuscular injection of the vaccine. Electroporation can facilitate cellular uptake and has been shown to enhance the immunogenicity and efficacy of DNA vaccines in pre-clinical animal models.⁵ In fact, the DNA-growth hormone vector used to promote growth and reduce mortality in pigs (see above) is delivered by electroporation. Electroporation has also been used to enhance the immunogenicity of a DNA vector expressing a prostate tumor-tetanus toxin fusion protein in a recent Phase I trial.¹²¹ Although the pain associated with electroporation was reported to be brief and acceptable, the universal tolerability of electroporation systems for humans has not been determined. Although the initial promise of DNA vaccines has not yet been fulfilled in humans, recent advances to enhance the potency of these vectors by modifying the plasmid backbone, the transgene, the vaccine formulation or the delivery system, and their potential use in prime-boost regimens may eventually make DNA vectors a viable vaccine platform.

Viral vectors. Since malaria vaccines based on viral vectors express high levels of antigen directly in infected cells of the host, antigen is processed and presented to professional antigen

presenting cells in a manner that is similar to natural infection. This provides a key advantage over protein subunit-based vaccines and is one of the reasons that viral vectors induce robust T cells responses. Also, appropriate folding of the delivered antigen is facilitated by synthesis within the vaccinated individuals' cells, resulting in the induction of functional antibody responses. In addition, as mentioned above, viral vector delivery systems provide adjuvant activities along with antigen delivery. The leading viral vectors for malaria vaccine development are based on poxvirus and adenovirus, because of their ability to induce potent antigen-specific immune responses in people, as well as their capacity to deliver multiple antigens. Other viral vectored vaccines based on alphavirus¹²² and yellow fever virus^{123, 124} have induced protective responses in animal models and are also being explored.

Poxvectors. Vectors based on attenuated vaccinia virus NYVAC and modified vaccinia Ankara (MVA), as well as fowlpox have been developed for malaria vaccines. In murine models, vaccination with a NYVAC vector expressing *P. berghei* CSP induced CD8⁺ T cell-mediated protection in mice.¹²⁵ These results prompted the generation of NYVAC-Pf7, a genetically engineered deletion mutant of the Copenhagen strain of vaccinia virus that expresses seven *P. falciparum* antigens derived from multiple stages in the parasite life cycle [CSP, SSP2, LSA1, MSP1, AMA1, serine repeat antigen (SERA), and Pf25].¹²⁶ In the first clinical trial of a poxvirus-based malaria vaccine, NYVAC-Pf7 was well tolerated at doses of 1×10^7 and 1×10^8 plaque forming units and partially protective. One volunteer was completely protected and there was a significant delay in the time to parasite patency in volunteers that received either the low or high dose of vaccine compared with unvaccinated controls.¹²⁷

Prime-boost strategies have significant advantages for malaria vaccine development.¹²⁸ Poxvirus vectors, FP9 and MVA, have been used together and with DNA and adenovirus vectors in prime-boost vaccination regimens. These studies demonstrated that the prime-boost approach induced more robust immune responses and superior protective responses in rodent models than either regimen alone. In clinical studies, DNA prime-MVA boost studies employing ME-TRAP antigen¹²⁹ induced higher levels of T cells and delays to parasitemia following challenge compared to either modality alone.^{130, 131} Prime-boost vaccination with FP9-MVA (also using ME-TRAP) was safe, immunogenic and partially protective in malaria-naïve volunteers receiving experimental challenge, providing sterile protection in a few cases.^{132, 133} The immunogenicity of the FP9-MVA (ME-TRAP) prime-boost vaccine was reduced in adults¹³⁴ and children¹³⁵ in malaria endemic regions in Kenya and provided no evidence of efficacy. The reasons for this discrepancy are not known. (For more details on this approach see the review in this edition by Hill et al., p. 78).

Adenovirus vectors. Adenovirus vectors can be engineered to induce broad and protective T cell and antibody responses in animal models, ranging from mouse to non-human primates.¹³⁶⁻¹⁴⁶ Protection from malaria has been observed using adenovectors that express the *P. yoelii*^{147, 142, 148} or *P. berghei*¹⁴⁹ CSP antigen in murine models. An Ad5-based HIV vaccine induced antigen-specific CD4⁺ and CD8⁺ T cell and antibody responses in a majority

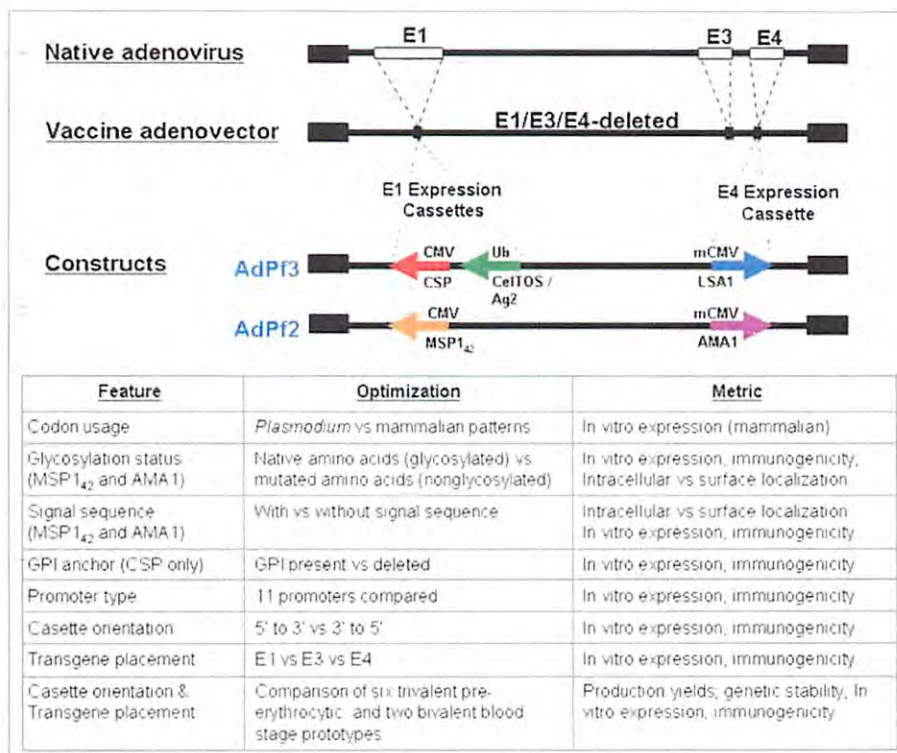


Figure 3. AdPf5. AdPf5, a pentavalent multistage malaria vaccine candidate comprising a trivalent pre-erythrocytic stage adenovector and a bivalent blood stage adenovector, exemplifies many of the molecular design features discussed in this review. The trivalent vector (AdPf3) expresses three *P. falciparum* (3D7 strain) pre-erythrocytic antigens: PfCSP, PfLSA1 and PfAg2 (CellTOS). The bivalent vector (AdPf2) expresses two *P. falciparum* (3D7 strain) blood stage antigens: PfMSP1₄₂ and PfAMA1. Both vectors are based on an E1/E3/E4-deleted Ad5 vector platform; because both E1 and E4 are required for replication, the vector is multiply replication incompetent. While E3 is not required for replication, its partial deletion increases the carrying capacity of this vector. All five genes, as well as their placement into the adenovector backbone, have been evaluated in multiple configurations in order to optimize several important characteristics. These include: codon optimization (all antigens), inclusion of a heterologous secretion signal (MSP1₄₂) and deletion of GPI anchor (CSP). The two blood stage antigens were also evaluated to determine the effect of N-linked glycosylation on induction of functional antibody responses. Mutations in N-linked glycosylation sites did not induce better antibody responses in rabbits and were not included in this malaria vaccine candidate. Different promoters are used for each transgene to prevent homologous recombination events. Promoters were selected and positioned in the adenovirus genome for optimal antigen expression in transduced cells and induction of robust antigen-specific T cell responses in mice and growth inhibitory antibody responses in rabbits. These assays showed that both vectors induced robust T cell and antibody responses to each of the five antigens in mice and the bivalent blood-stage vector induced growth inhibitory antibody responses to each antigen in rabbits. Both vectors grow to high titers in 293-ORF6 cells under serum free suspension conditions and are genetically stable. The AdPf5 vaccine comprises two adenovector constructs, AdPf3 (pre-erythrocytic stage vector) and AdPf2 (blood stage vector), which are mixed together prior to intramuscular administration. Key: CMV, human cytomegalovirus promoter; mCMV, murine cytomegalovirus promoter; Ub, ubiquitin promoter. Arrows indicate the direction of transcription.

of volunteers.¹⁵⁰ Similarly, we observed robust CD4⁺ and CD8⁺ T cell responses in the first clinical trial of an adenovirus serotype 5 vector (Ad5)-based malaria vaccine candidate expressing CSP and AMA1 antigens. As both CD4⁺ and CD8⁺ T cell responses have been implicated in the sterile protection observed following immunization of volunteers with radiation-attenuated sporozoites, these findings suggest that adenovectors might be able to induce the requisite immune responses for protection against malaria.

Multi-gene expressing adenovectors for malaria vaccine development. Adenovirus vectors can be generated that express multiple proteins from different expression cassettes located within the adenovirus genome.¹⁵¹⁻¹⁵⁴ Vaccines based on these vectors have advantages from both potency and cost-of goods perspectives. Moreover, this multi-gene expression technology is

highly flexible, facilitating rapid substitution of different antigens into the multi-gene expressing adenovector after it is developed. A key requirement is that vectors must have sufficient capacity to express multiple antigens. We have generated a multiply deleted Ad5 vector with room for incorporation of up to 9Kb of foreign sequences, by deletion of three Ad5 early regions, including complete deletions of both early regions 1 and 4 (E1 and E4) and a partial deletion of region 3 (E3). E1/E3/E4-deleted vectors can be produced in 293-ORF6 cells, which provide complementation for both E1 and E4 functions¹⁵⁵ and are suitable for cGMP manufacturing of adenovector preparations free from replication competent adenovirus (RCA).¹⁵⁰ These multiply-defective (E1/E3/E4-deleted) vectors express much lower levels of adenovirus genes following infection relative to an E1/E3-deleted vector. Consequently, immunization of volunteers with E1/E3/

E4-deleted vectors did not induce an expansion of adenovirus-specific CD4⁺ T cells,¹⁵⁶ a characteristic that could have important safety advantages. Recently, we have generated Ad5 vectors that express high levels of three different pre-erythrocytic stage antigens [CSP, LSA1 and cell traversal protein of ookinets and sporozoites (CelTOS)] from three different expression cassettes located in the E1 and E4 regions of the vector genome (see Fig. 3). In preclinical studies in mice, these vectors induced T cell and antibody responses specific for each of these antigens and the magnitude of these responses was similar to that of control Ad5 vectors that expressed only one antigen.⁶ We are currently applying this multi-gene expression technology to alternative serotypes of adenovirus where the prevalence of neutralizing antibodies (NAb) in human populations is very low.

Adenovector neutralizing antibodies (NAb): prevalence and impact on vaccine safety and efficacy. Adenovirus vectors derived from Ad5 have been a leading platform for the generation of molecular vaccines. However, forty to sixty percent of the United States population possesses NAb to Ad5^{99, 157} and the frequency is even higher in Africa, where malaria is endemic.¹⁵⁸⁻¹⁶⁰ In animal models including mice¹⁶¹ and non-human primates,¹⁶² prior exposure to Ad5 diminished T cell responses to the vaccine antigens delivered using Ad5 based vaccines. As expected, due to the subtype specificity of adenovirus NAb,¹⁶³ pre-existing Ad5 NAb were not effective in reducing vaccine responses to other adenovirus vaccines based on alternative human^{157, 164} or simian serotypes.¹⁶⁵⁻¹⁶⁸

Reports addressing the impact of pre-existing Ad5 neutralizing antibody on adenovector vaccine immunogenicity in the clinic (with HIV vaccines) indicated that volunteers possessing high titers of Ad5 neutralizing antibody were capable of mounting significant antigen-specific humoral and cellular responses.^{150, 169, 170} However, the magnitude of the T cell responses in individuals with pre-existing Ad5 NAb was approximately three-fold lower than those of the Ad5-seronegative volunteers.

The most conservative path forward for adenovirus-based vectors is to advance vaccines based on alternative serotypes of adenovirus where the prevalence of NAb is low. However, finding a highly immunogenic substitute for Ad5 has not been straightforward. To date other serotypes of adenovirus (Ad35, Ad11, Ad26, Ad48, Ad49, Ad50 and chimpanzee adenovectors) have generally under-performed Ad5 vectors in terms of induced immunogenicity to their payload antigen.^{97, 147, 161, 164, 171, 172}

Capsid-modified adenovectors. An alternative strategy for avoiding preexisting Ad5 NAb is to modify the NAb binding sites on the Ad5 capsid. The majority of serotype-specific neutralizing antibodies recognize determinants on the hexon and fiber capsid proteins,^{160, 173, 174} and hexon-specific neutralizing antibodies appear to be the most prevalent and potent in vivo.¹⁶⁴ Epitopes targeted by these hexon-specific neutralizing antibodies have been mapped to the hypervariable regions (HVR) of the hexon protein^{174, 175} contained within exposed loops at the surface of the capsid. Roberts et al., 2006 demonstrated that it is possible to replace each of the 9 HVR of the Ad5 hexon with those derived from Ad48, a rare adenovirus serotype.¹⁷⁶ Induction of transgene-specific immune responses was unaffected by Ad5-specific NAb in murine or non-human primate vaccine models.

GenVec, Inc. has developed a hexon-modified adenovector vaccine platform for malaria vaccine development that avoids preexisting anti-adenovirus immunity prevalent in human populations by replacing the Ad5 HVR sequences with those derived from Ad43. This Ad5 hexon-modified vector is not neutralized by hexon-specific Ad5 neutralizing antibodies in vitro using sera from mice, rabbits and human volunteers. Furthermore, the hexon-modified adenovector that expresses PyCSP is as immunogenic in mice as an unmodified Ad5 vector and can induce robust T cell responses in mice with high levels of preexisting anti-Ad5 Nab. In addition, the hexon-modified vector effectively boosted Ad5 vector primed T cell and antibody responses.¹⁷⁷

Adenovirus capsids can also be modified to increase the immunogenicity of the vector. We have demonstrated that the introduction of a DC targeting ligand into the fiber improved DC transduction and antigen-specific T cell responses.⁹⁸ Also, B cell epitopes can be inserted into the hexon, fiber and penton base and displayed on the capsid to induce protective immune responses in mice.^{146, 178-180}

Bacterial Vectors. Currently, there are three commercially available live bacterial vaccine vectors: *Salmonella enteric* serovar Typhi Ty21a, *Vibrio cholera* CVD 103-HgR, and *Mycobacterium bovis* BCG. Live attenuated bacteria are an attractive vectored platform for expression, delivery and eliciting host immunity to foreign antigens. The bacterial vector itself mimics natural infection in the host, exhibiting tissue tropisms that enable the targeting of vaccine antigens to various cellular compartments, thereby inducing mucosal, humoral and cellular immune responses. This approach has been widely used for expression and delivery of heterologous targets derived from various bacteria, fungi, viruses and parasites in clinical and veterinary applications, particularly for gastrointestinal, genital and respiratory pathogens. The specific advantages of this approach include (1) the relative low cost and potential for large scale manufacture of target antigen by the "bacterial factory," (2) the presence of natural immunostimulatory molecules, such as LPS and flagellin, thus obviating the requirement for inclusion of classical adjuvants (see section on TLR agonists), (3) sensitivity to antibiotics for eradication if indicated and (4) tropism for dendritic cells and macrophages. Concomitantly, safety considerations for live attenuated bacterial vaccines focus on the possibility of undesirable systemic dissemination, toxic bacterial byproducts (LPS), transmission through contact to either healthy or immune compromised recipients, and/or the potential for environmental contamination.

Vaccination using attenuated or killed bacterial vectors that serve as antigen vehicles can provide a dual-pronged immunization strategy -- to elicit protective responses against the target antigen and the vehicle itself. These attenuated bacterial vaccine vectors can stimulate potent immune responses by inducing the production of cytokine cascades, including tumor necrosis factor alpha (TNF α), IFN γ , IL-12 and other proinflammatory mediators such as NO.¹⁸¹ The type of immunity elicited by these bacterial vectors is strongly dependent on the bacterium itself, the route of delivery, as well as other biological indicators

associated with the target antigen. For example, the intracellular pathogen *Listeria monocytogenes* induces both mucosal and systemic immunity. In murine models, using either *E. coli* β -gal expression or oral administration of *L. monocytogenes* expressing HIV-1 gag, both mucosal and systemic CD8⁺ cellular immune responses were detected against the target antigens.^{182, 183}

Nearly two decades ago, an attenuated *Salmonella typhi* was used to elicit a human serologic or cytotoxic T lymphocyte response to a foreign target antigen, *P. falciparum* CSP.¹⁸⁴ The attenuated *S. typhi* vaccine strain CVD 908, harboring deletion mutations in *aroC* and *aroD*, was shown to be well-tolerated and highly immunogenic, eliciting impressive serum antibodies, mucosal IgA and cell-mediated immune responses. A decade later, when *P. falciparum* MSP1₁₉ was genetically fused to fragment C of tetanus toxin and then expressed within attenuated *S. typhi* CVD 908, under nonreductive intracellular bacterial conditions, chimeric C-MSP1₁₉ did not fold to form the properly disulfide bridged epidermal growth factor (EGF)-like domains of MSP1. Disulfide dependent monoclonal antibodies failed to recognize these conformational domains in immunoblots under non-denatured conditions on bacterial lysates. These results demonstrate that proper targeting of the antigen to the appropriate cellular compartment is required.¹⁸⁵ In a recent report, *S. enterica* serovar Typhi strains were used for expressing and exporting truncated *P. falciparum* CSP B-cell and T-cell immunodominant epitopes fused to *S. enterica* serovar Typhi cytolysin A (ClyA), which were then used in a heterologous prime-boost approach with a DNA-vectored vaccine. Interestingly, mice that were primed with serovar Typhi expressing CSP epitopes and boosted with DNA plasmid induced high antibody titers to NANP, predominantly IgG2a, and high frequencies of IFN γ producing T cells, while mice receiving only DNA vectored vaccine did not induce any antibodies.¹⁸⁶ In a recent published report, investigators showed that the genetic detoxification of the *S. enterica* serovar Typhimurium to improve its safety profile led to a serendipitous enhancement in antigen-specific immune responses.¹⁸⁷ Additional modifications may lead to safer and more effective *S. typhi* vaccine platform.

Other bacterial vectors have been used for targeting malarial epitopes, including a recombinant *Mycobacterium bovis* bacille Calmette-Guérin (rBCG) vector expressing epitopes from *P. falciparum* EBA 175 and CSP (F2R(II)EBA and (NANP)3 respectively) as well as two T cell epitopes of the *M. tuberculosis* ESAT-6 antigen.¹⁸⁸ Immunized BALB/c mice showed an enhanced antibody response to all IgG subclasses, as well as an enhanced splenocyte proliferative response against both malarial epitopes. Combining immunization platforms, for instance, a prime-boost vaccination strategy that encompasses the benefits of BCG with that of a potent boosting agent like MVA, could provide a strategy not only to broaden anti-mycobacterial immunity, thus reducing the burden of TB disease, but also to target malaria, HIV or other concurrent pathogenic infections.¹⁸⁹ Specifically for TB, when administered in healthy adults, this vaccination strategy induced good CD4⁺ T cell responses with the capacity to produce effector cytokines and to proliferate following antigen stimulation.

Previously, *ompA* genes of *Escherichia coli* and *Shigella dysenteriae* have been used to construct enterobacterial surface expression vectors for foreign genes. These fusion constructs have been widely used as a vector system in experimental models and have been used to transfer target antigens, such as β -galactosidase, foot-and-mouth disease virus (FMDV) and malaria antigens, to the bacterial surface as insertions into OmpA. Several strains of attenuated *Shigella*, such as *S. flexneri* 2a, have been developed and used in various human clinical trials with good tolerability.¹⁹⁰ Alternatively, food-grade gram-positive bacteria such as *Lactococcus lacti* have advantages over other attenuated bacterial vaccine delivery vehicles primarily because of their inherently greater safety profiles. *P. falciparum* merozoite surface protein-2 (MSP2) expressed in recombinant *L. lactis*, either intracellularly or covalently anchored to peptidoglycan on the cell wall, elicited serum IgG antibodies that reacted with native MSP2 on the surface of *P. falciparum* merozoites, as detected by immunofluorescence.¹⁹¹

A eukaryotic vector has also been used to deliver malaria antigens. In one published study, recombinant attenuated *Toxoplasma gondii* expressing the *P. yoelii* CSP proved highly effective in priming for CD8⁺ T cell-dependent protective immunity in mice.¹⁹²

Virus-Like Particles

Virus-like particles (VLPs) are non-infective, self-assembling viral envelope proteins that are devoid of genetic material. They have features relevant to both antigen design and to antigen delivery, on the one hand providing a macromolecular scaffolding for the array of antigen, and on the other triggering host immunity by taking advantage of natural immunostimulatory properties of the virus particles that they mimic.

VLPs are highly repetitive and ordered structures that retain their morphology similar to infective particles and have been shown to stimulate both potent humoral and cellular immune responses, although they are most effective at stimulating humoral immunity.¹⁹³ Two examples of successful application of this approach are the hepatitis B virus (HBV) vaccine, a formulation prepared from VLPs assembled in yeast expressing the hepatitis B surface antigen (HBsAg), and the human papillomavirus (HPV) vaccine, a quadrivalent formulation also prepared from VLPs assembled in yeast expressing the L1 major capsid protein of HPV strains 6, 11, 16 and 18. Each of the vaccines targets the homologous infectious agent (the agent from which the structural components of the VLP are derived). VLPs made by the expression of recombinant capsid proteins in baculovirus-infected insect cells are also under study as potential subunit vaccines against norovirus, rotavirus, hepatitis C virus, hepatitis E virus, and human immunodeficiency virus (all homologous targets).¹⁹⁴⁻¹⁹⁷

Molecular design and engineering have provided the means for converting these VLPs into platforms for presenting heterologous antigens. Principally, the development of such platforms requires having sufficient structural information to allow for the rational design and incorporation of foreign antigens into self-organizing capsid proteins without compromising the capacity

to assemble into a VLP. Two basic approaches are used. The first is the creation of a fusion gene expressing the heterologous antigen in frame at an appropriate point within the envelope protein to result in display on the VLP surface. The resulting product may require mixing with unmodified envelope protein to achieve stable self-assembly, and because of structural constraints may only be able to tolerate the introduction of short foreign peptide sequences, such as an antigenic epitope. The second strategy is to allow the unmodified envelope protein to assemble into VLPs as the first step, and then heterologous antigen is coupled to the particle as a second step. The latter technique generally does not interfere with particle stability, allowing decoration with large protein domains and even full-length proteins.¹⁹⁸ Whichever technique is used, the density of the heterologous array on the surface is critical to potency. It is hypothesized that array density determines the efficiency with which the receptors of inquiring lymphocytes are cross-linked. As a result, low density arrays, such as the RTS,S vaccine, are not ideal for inducing immunity in the absence of adjuvant. The size of the VLP, which varies from 22 to 150nm, is less important for achieving potency than the array density.

The list of VLPs that have been studied is extensive, and includes those assembled with the capsid proteins from mammalian (HBV, HPV, parvovirus B19), avian (Flock House-virus, FHV), plant (papaya mosaic virus, cucumber mosaic virus), and yeast (TY-1, L-A) viruses, as well as bacteriophage (MS2, Q-beta). The two VLPs studied most extensively as a platform for malaria vaccines are based on the hepatitis B core (c) and surface (s) proteins.

Hepatitis B virus core protein (HBc) was demonstrated to be an effective malaria vaccine platform in preclinical models, where high levels of anti-CS repeat antibodies protected animals from malaria challenge.¹⁹⁹ In a recent nonrandomized, non-blinded Phase I safety study using ICC-1132 (Malariavax), a recombinant VLP comprised of hepatitis B core antigen engineered to express the central repeat regions from *P. falciparum* CSP protein, an immunodominant B [(NANP)₃] epitope, an HLA-restricted CD4 (NANPNVDPNANP) epitope and a universal T cell epitope (amino acids 326–345) was found to be well tolerated, but poorly immunogenic when formulated with alhydrogel.²⁰⁰

Alternatively, the *P. falciparum* CSP-based vaccine, RTS,S, developed by GSK, is composed of a hepatitis B virus surface antigen, which contains the CSP repeat and C terminal region (amino acids 207–395) of the *P. falciparum* NF54 isolate (3D7 clone). RTS,S is the most advanced malaria vaccine, currently in pivotal Phase 3 efficacy trials in sub-Saharan Africa. In combination with GSK proprietary adjuvant systems, this vaccine has demonstrated significant protective efficacy against malarial disease and severe malaria.^{201,202} Adjuvanted RTS,S has been shown to induce both a strong antibody response and stimulate Th1 cells—a subset of helper T cells—that participate in cell-mediated immunity.²⁰³ It is the relative success of RTS,S that has provided impetus for researchers to consider, develop and evaluate alternative particulate-based vaccine platforms.

Another particle approach, virosomes, represents a variation on VLPs. Virosomes are made up of liposomes containing viral

envelope proteins and have been previously developed for many enveloped-viruses, i.e., semiliki forest, measles, rubella, herpes simplex, rabies, sindbis, Epstein-Barr, HIV and vesicular stomatitis virus. All influenza virosomes are unilamellar vesicles having a diameter of 100–200nm depending on lipid composition. The major advantages of virosomes include their immunogenicity in the absence of adjuvants and their relatively simple production compared to traditional recombinant protein-based processes. Virosomes are thought to enhance immune responses against co-delivered target antigens at multiple levels. Virosomes can interact with APCs and B lymphocytes to present the target antigen to T lymphocytes to induce both antigen-specific B cell and CD4⁺ T cell responses. Optimally, virosomes can act as carriers of B cell epitopes, but are also promising for the delivery of carbohydrates, DNA, siRNA, and drugs.

Virosomes can be derived solely from virion lipids or from virion plus supplementary lipids (e.g., Immunopotentiating Reconstituted Influenza Virosomes, IRIV). The final composition of lipids forming the IRIV can impact not only the physiochemical properties of the resultant virosome, but also immunological properties. IRIV act as antigen carriers through encapsulation or direct association, providing significant flexibility for formulation. Pevion Biotech has developed optimized formulations of IRIV designed for specific applications (reviewed in Moser et al.).²⁰⁴ AMA 49-CPE is an IRIV that was evaluated as the carrier and adjuvant system for a *P. falciparum* vaccine that targets both the exo-erythrocytic and erythrocytic stages based on two malaria peptide antigens (AMA1 and UK39, a circumsporozoite protein) previously shown to induce parasite inhibitory activities.²⁰⁵ Although this vaccine approach was found to be safe and highly immunogenic in a placebo-controlled, randomized clinical Phase 1a safety study conducted at the University Hospital of Basel,²⁰⁶ no sterile protection was observed against a homologous sporozoite challenge in a subsequent clinical Phase 2a trial.²⁰⁷ However, some evidence for the development of blood stage immunity was reported, as shown by a reduction in parasite growth rates and the observation of crisis forms in the blood from some volunteers. A Phase 1b study to evaluate the safety and immunogenicity of this vaccine in Tanzanian adults and children has been completed and final results are awaited (a collaboration between the Bagamoyo Research and Training Unit and the Swiss Tropical Institute).

Another variation on molecularly designed particulate vaccines is represented by self-assembling polypeptide nanoparticle (SAPNs) (Fig. 4 and Box 2). As with VLPs and virosomes, the repetitive display of B cell epitopes on the nanoparticle may lead to the induction of a strong humoral immune response. Additional engineering to include the pan-DR T cell epitope, PADRE, or other relevant helper sequences into the peptide design may influence the immunogenicity of the nanoparticle constructs. These peptide nanoparticles have a reported icosahedral symmetry which mimics viral protein capsids, but can be specifically designed to vary in size and the density of the surface array. Since these peptide nanoparticles are recombinant peptide-based vaccines, the safety risks of live-attenuated vaccines are avoided. In studies using an experimental murine model of malaria, SAPNs

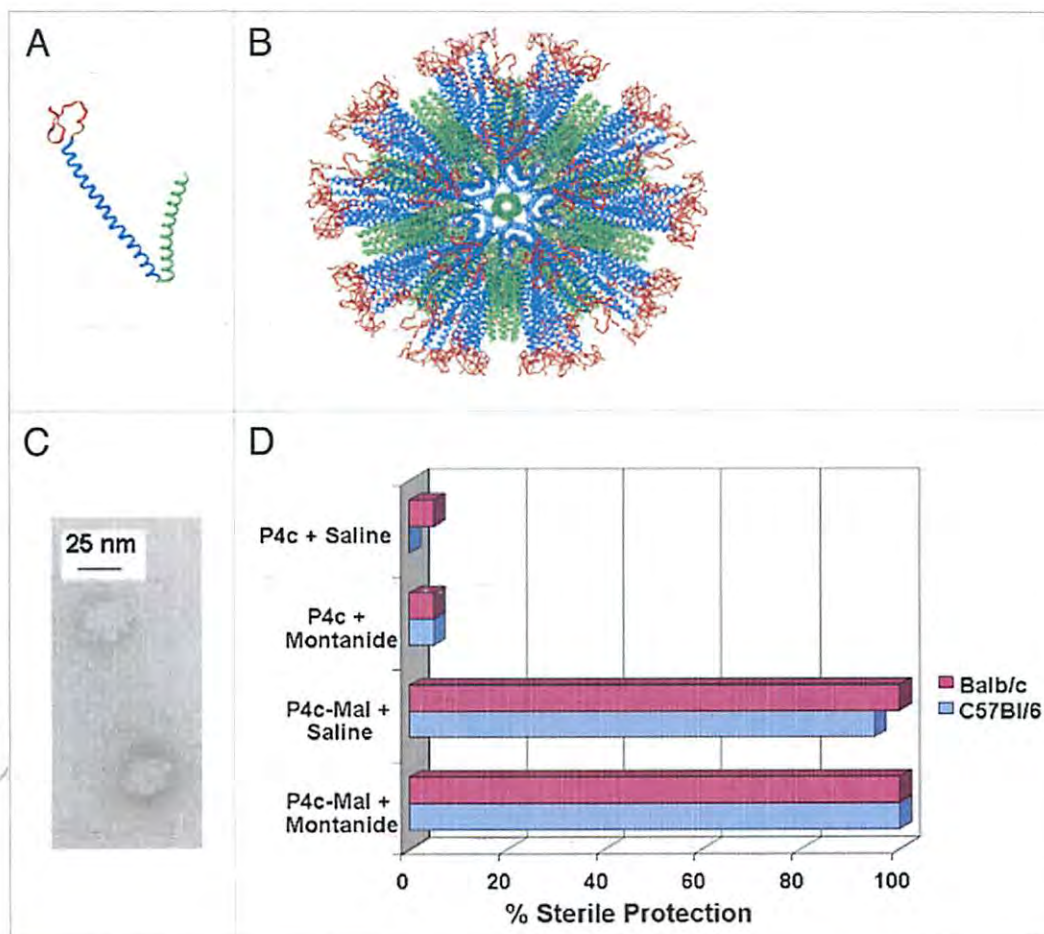


Fig 4. Self-assembling polypeptide nanoparticles (SAPN). (A) Monomeric building block of P4c-Mal composed of a modified pentameric coiled-coil domain from cartilage oligomeric matrix protein (COMP) (green) and a trimeric de novo designed coiled-coil domain (blue) which is extended by the sequence of *P. berghei* CSP repeat region, (DPPPPNPN)₂D (red). (B) Computer model of the complete peptide nanoparticle (P4c-Mal), containing the repeat region of *P. berghei* CSP, with icosahedral symmetry. The calculated diameters of these particles are about 25nm and have a molecular weight of 14.8 kDa. (C) Transmission electron microscopy image of P4c-Mal nanoparticles at 242 000 \times . The sample was negatively stained with 1% uranyl acetate. Sample concentration was 0.076 mg/mL and the buffer was 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol. Nanoparticle size ranges are from 25 to 30 nm. (D) BALB/c and C57BL/6 mice were immunized three times with P4c-Mal or P4c (empty) SAPNs at 10 μ g dose in the presence or absence of adjuvant, Montanide ISA 720, at two week intervals and challenged with 4000 (for BALB/c) and 1000 (for C57BL/6) *P. berghei* sporozoites by intravenous injection two weeks post final immunization. SAPN P4c-MAL induced approximately 100% sterile protection in both BALB/c and C57BL/6 mice regardless of adjuvant inclusion.²⁰⁸ These excellent protection results support the further development and evaluation of SAPNs for malaria.

designed to express peptide epitopes from the repeat region of the *P. berghei* CSP provided nearly 100% protection against sporozoite challenge with *P. berghei* sporozoites without the requirement for additional adjuvant (Fig. 4).²⁰⁸ Furthermore, a SAPN displaying the *P. falciparum* CSP repeat epitope completely protected mice from challenge using a *P. falciparum* CSP transgenic *P. berghei* parasite without the requirement for additional adjuvant (D. Lanar, personal communication).

Safety of Molecular Vaccines

Establishing safety is the most important development objective for a malaria vaccine assuming that it offers significant protection. Because the intended target populations are healthy individuals,

including small children, infants, travelers, pregnant women and females entering puberty, tolerability should be acceptable and the safety profile excellent.

The clinical assessment of molecular vaccines, as with any vaccine, aims to identify adverse events stemming from the injected material. Such hazards fall into two categories—adverse events that are anticipated, given what is known about the vaccine, its formulation and mechanism of action, and adverse events that are unexpected. The occurrence of the former can be specifically sought in clinical trials through testing or other design features built into the trials. The latter cannot be anticipated and dictates that early studies should comprise small numbers of volunteers subject to intense follow-up in order to safely identify unknown risks.

Box 2. Self-assembling peptide nanoparticles: Novel vaccine platforms based on virus-like particles are gaining increased interest in the field of vaccine development. Peptide-based nanoparticles represent an ideal repetitive epitope display system and a platform for the development of highly tailored recombinant vaccines. These peptide nanoparticles have a regular polyhedral symmetry that mimics the icosahedral architecture of viruses. As such, epitope display approaches have been shown to strongly increase the immunogenicity to a given epitope. The fundamental structure of SAPNs is derived from the molecular design of a monomeric linear polypeptide having about 100 amino acids (Fig. 4). Each linear polypeptide is comprised of a pentameric and a trimeric coiled-coil oligomerization domain joined by a two-glycine residue flexible linker. These alpha-helical coiled-coils represent a common protein oligomerization motif stabilized through hydrophobic interactions occurring along their coiled-coil interface. Both N- and C-terminal ends of the polypeptides in the assembled complex are exposed on the surface, allowing for presentation of B cell epitopes in a repetitive antigen display. Peptide epitopes of B cell, T helper, or CTL specificity can be molecularly incorporated into polypeptides by the cloning of their nucleotide sequences into an expression plasmid used to express the polypeptides. Following expression in *E. coli*, the protein can be purified, refolded, and assembled into nanoparticles having a diameter of approximately 25 nm. By taking advantage of the increasing knowledge of this structural class of protein motifs, a growing number of biological and therapeutic applications are being considered.²⁰⁸

Molecular vaccines based on genetic approaches potentially entail additional hazards beyond those of the injected material (vaccine substance), resulting from the fact that RNA transcripts are synthesized *in vivo* following injection and host cell entry, and these in turn lead to the expression of protein products, including both transgene antigens and incidentally expressed vector proteins. Because these products are expressed intracellularly, the safety of a given genetic vaccine cannot be demonstrated by clinical studies of the exogenous protein encoded by the transgene, which will remain primarily in the interstitial tissues following administration. Thus, a candidate malaria antigen that has been studied in humans as a recombinant protein requires new toxicology studies if subsequently developed as a DNA- or virally-vectored vaccine.

Each molecular vaccine platform carries its own particular safety risks. For example, viral proteins and nucleic acids present in viral vectors display a variety of pathogen-associated molecular patterns that trigger specific host pattern recognition receptors to activate the innate immune system. Viral proteins may trigger TLR 2 and 4 receptors, viral RNA TLR 3, 7 and 8 receptors, and viral DNA the TLR 9 receptor. Each platform can be expected to induce different patterns of immune activation and cytokine release based on its effects on the innate immune system. As an example of side effects consistent with these known pathways, we have observed that most recipients of our Ad5-vectored malaria vaccine, NMRC-M3V-Ad-PfCA, demonstrate transient dose-dependent reductions in circulating neutrophils and lymphocytes, likely due to margination and diapedesis into the interstitial space of peripherally circulating white cells in response to the danger signal and resulting cytokine cascade engendered by the viral capsid or its DNA.²⁰⁹ This occurs during the first few days following administration of the vaccine and parallels published data on the side effects of CpG 7909, a TLR9 agonist, when administered subcutaneously.²¹⁰ Likely also associated with

the intrinsic inflammatory properties of the vaccine components, adenovirus-vectored vaccines cause fever and other systemic reactions, generally with doses of 1×10^{11} particle units or higher. Additional safety concerns are discussed in Box 3.

In addition to these side effects that are consistent with our understanding of the vaccine vector, vaccines may cause a range of unexpected, idiosyncratic reactions based on environmentally-induced or genetic differences among individual recipients which are difficult to predict. For example, the poxvirus platform became the subject of concern when sporadic cases of myocarditis were linked to the administration of replicating vaccinia virus as part of a smallpox immunization campaign (CDC 2003). However, a subsequent study of volunteers receiving MVA and other non-replicating poxvectors indicated that this concern did not extend to these related poxvirus platforms. Most recently, the Merck STEP trial suggested a link between naturally acquired immunity to Ad5 (acquired following exposure to wild-type virus earlier in life) and an increased risk of HIV acquisition in uncircumcised men receiving the Ad5-vectored HIV vaccine (see Box 3).²¹¹ No plausible mechanism to explain this finding has been established since the STEP trial results were announced in November, 2007. The finding itself remains unconfirmed by other studies, as investigators search to identify covariates affecting the results of the STEP trial that may have contributed to this finding.

Particularly because of unpredicted side effects such as those just described, each candidate malaria vaccine based on molecular approaches needs to be examined carefully for toxicity, although when a platform is shared by two vaccines (e.g., identical DNA backbones, adenovirus or poxvirus vector systems, etc.), it is appropriate to cross-reference nonclinical and clinical studies in support of safety. This is true, for example, of the cell substrate used to produce a vaccine: once safety is demonstrated and the cell line is qualified for production of vaccines for use in healthy humans (such as for the PER.C6 or 293-ORF6 cell lines used to produce adenovirus vectors), retesting the cell line for each vaccine may not be required. In addition to careful evaluation in nonclinical studies, the vaccine itself should be designed with safety considerations prominently in mind. For example, many malaria antigens contain sequences sufficiently similar to human sequences (e.g., thrombospondin-like domains^{212, 213}) that it is appropriate to conduct BLAST searches^{††} against the human genome in order to avoid sequences in the vaccine that, due to homology, might favor integration into the host genome or the breaking of self-tolerance.

Once a new vaccine or previously-tested vaccine based on a new cell substrate has demonstrated safety in appropriate nonclinical studies and is advanced to clinical testing, it is important to evaluate safety in a very small number of individuals, with a delay before commencing further immunizations. An example where this procedure was not followed involved the administration of TGN1412, a humanized monoclonal antibody designed as an agonist to the CD28 receptor on T lymphocytes. Six of the eight

^{††} Basic Local Alignment Search Tool

Box 3. Adenovector Safety: Each molecular platform, and potentially each molecular vaccine, is characterized by theoretical safety concerns that may or may not turn out to be significant *in vivo*. These concerns must be rationally addressed prior to clinical testing, potentially by conducting nonclinical studies, and then monitored closely, to the extent possible, in Phase I studies. Here are several concerns relating to adenovirus vectors:

Integration potential: Although adenoviruses infect a wide range of cell types, their genome remains episomal in the infected host cell and they lack native integration machinery. A report from the European Medicines Agency classified adenovirus as a non-integrating vector.²⁵² However, studies have shown that replication-incompetent adenoviral vectors can randomly integrate into host chromosomes, at frequencies of 10^{-2} – 10^{-5} per infected cell, when studied *in vitro* in replicating cells where active selection was used to detect these events.^{253, 254} This low rate has limited the usefulness of unmodified, non-replicating adenoviruses as vehicles for effecting permanent gene transfer, but represents an advantage for their use as vaccine vectors where long-term expression is not required. The risk of insertional mutagenesis in neighboring genes resulting from integration events would be expected to be lower than the rate of integration, since only a small proportion of integration events would be expected to result in mutagenesis.

Latency and transformation: Wild-type adenoviruses can remain latent in tissues and have been detected in tumors as well as in normal tissues, although there is no definitive evidence that vectors such as Ad5 (Group C adenoviruses) play a causative role in human cancer,²⁵⁵⁻²⁵⁹ nor is there evidence of germline transduction.^{260, 261} Adenoviruses are also reported to have transforming (immortalizing) potential *in vitro*, including Group C adenoviruses.^{262, 263} One approach to addressing this concern is to delete the regions of the virus associated with transformation, notably the E1A and E1B genes.²⁶⁴ Most adenovectors currently in the clinic have the E1 region of the virus removed and replaced with the expression cassette for the transgene. The AdP15 (Fig. 3) vaccine also has the E4 and portions of the E3 genes removed.

Merck STEP Trial: The findings in the STEP trial (see text and Buchbinder et al.²¹¹) raise central questions about the safety of adenovirus vectors. Do they indeed increase the risk of HIV infection in those seropositive for anti-Ad neutralizing antibodies? Since the signal was detected only in uncircumcised men, could it relate, for example to the presence of CD4+ T cells and virus incubating under the foreskin following exposure through sexual contact, and thus remain restricted to this age, sex and risk group? Is the risk peculiar to Ad5 or would it apply to all viral vectors? Would the risk disappear with adenovectors that express significantly lower levels of viral proteins than the Merck vector, such as the E1/E3/E4-deleted AdP15 vector? Until these and additional questions are answered, it will be prudent to restrict the testing of adenovectors to seronegative individuals or, if seropositive, to those individuals who know and have control over their risk for HIV exposure.

healthy adult subjects receiving the injection in an initial immunization session developed multi-organ failure associated with a systemic inflammatory response syndrome and nearly died, despite reportedly unremarkable toxicity observed in rabbits and monkeys receiving up to 500 times the human dose.²¹⁴ Although this was a therapeutic monoclonal antibody, not a vaccine, the principle applies equally to molecular vaccines.

With these considerations in mind, molecular vaccines have generally been well-tolerated and safe in humans, both malaria-naïve research subjects tested in the US and Europe, and in malaria-exposed residents of endemic areas. This includes vaccines based on DNA,^{111, 112} poxvirus,^{127, 215-218} and adenovirus vectors (only results of adenovirally-vectored non-malaria vaccines are currently published^{150, 170, 219-221}) and prime-boost combinations of DNA

and poxvirus vectors or DNA and recombinant protein^{130, 222-224}. Protein-based malaria vaccines employing molecular approaches covered in this review have also generally proven safe and well-tolerated, including virus like particles,^{200, 225-227} virosomes^{206, 207} and bacterial vectors.¹⁸⁴ Theoretical concerns regarding the induction of autoimmunity (e.g., anti-double stranded DNA antibodies), integration into the host genome or germline, or persistence of transgene expression with resulting untoward effects, have not materialized. As the electroporation of DNA plasmids to increase cellular uptake and expression undergoes clinical testing, the question of integration into host genomic DNA must remain open, as electroporation may increase this risk.²²⁸ Viral vectors appear more reactogenic than naked DNA, but still carry an acceptable tolerability profile. The most extensively studied of virally vectored vaccine platforms, MVA, has been administered as a prevention for smallpox (thus without transgenes) to over 120,000 previously unvaccinated individuals, including elderly individuals, alcoholics and others with compromised immunity. Only a small proportion showed any systemic side effects, such as fever and there were no indications of serious reactions associated with the vaccine.²²⁹ In addition to these clinical studies, a substantial body of literature has been accumulated regarding animal toxicology, biodistribution and genomic integration (or lack thereof) of gene-based vaccines to support the good-safety profiles observed in humans.²³⁰⁻²³³

Molecularly-based adjuvants provide a different set of challenges. As with adjuvants used with protein-based vaccines, adjuvants for gene-based vaccines require extensive safety evaluation. After nonclinical studies indicated that the oligonucleotide CPG 7909 significantly enhanced the immunogenicity of the recombinant protein-based malaria vaccine, AMA1-C1,^{234, 235} subsequent clinic testing raised concerns regarding the frequency of allergic reactions⁶⁹ and this in turn led to identification and testing of less allergenic formulations.²³⁶ A major step in establishing safety of immunomodulatory adjuvant systems occurred with the co-administration of plasmids encoding human GM-CSF to healthy adults as an immunostimulant to improve the potency of the pentavalent MuStDO5 malaria vaccine (a mixture of five plasmids encoding pre-erythrocytic stage antigens).⁸⁶ Although a potentially adverse inflammatory response might have been anticipated following the injection of this human cytokine, the vaccine/adjuvant combination was well-tolerated, even if non-protective.²³⁷ One of the ways that the safety profile of this vaccine was assessed pre-clinically was to immunize mice with the vaccine in a standard toxicology study, but instead of co-administration with a plasmid encoding human GM-CSF, the mice were given a plasmid encoding murine GM-CSF to better reproduce a host-appropriate inflammatory response.²³¹ This illustrates that careful study design based on purported mechanism may be helpful in improving the predictive ability of non-clinical studies. The case of TGN1412 monoclonal antibody cited above provides another example where potential species-specific toxicity profiles misled investigators, with benign results in animal models failing to predict life-threatening toxicity in humans.²¹⁴ This principle is an important consideration for the immunomodulatory components of a vaccine, since many of these compounds act via a species-specific mechanism.

Potential rare side effects of molecular vaccines will remain a major concern, as their identification ultimately relies upon the larger sample sizes associated with Phase 3 testing and licensure, milestones not yet achieved by any gene-based vaccine. Post-marketing surveillance, key to this process, will be hampered for malaria vaccines, since many of the target populations reside in resource-poor countries lacking the capacity to adequately monitor and report serious adverse events post licensure.

Looking to the Future

Many vaccine developers are currently focusing on molecular vaccines. One of the most important reasons for this is their potential for improved safety and efficacy, especially for the difficult infectious disease targets, such as malaria, HIV and tuberculosis, where traditional approaches have failed to deliver effective vaccines or carry unacceptable safety risks. It is comforting for the customer and satisfying for the vaccine developer to conceive and build a vaccine based on known biological mechanisms and precisely defined molecular targets. This type of immunogen can be manufactured according to tightly controlled, reproducible processes, yielding a drug substance where nearly every attribute is defined. Ultimately, the comprehensive understanding intrinsic to a well-conceived molecular vaccine should improve both safety and efficacy, thereby meeting the major goals for vaccine developers.

Another important benefit of molecular vaccines is their agility. Knowing the mechanism of action allows the design of functional assays for monitoring vaccine efficacy that can promptly detect vaccine resistance by the pathogen and enable modification of the vaccine in a timely way to compensate. A re-engineered immunogen can be placed into the existing expression cassette, and the improved product manufactured by the same processes as the original product.

Ultimately, however, molecular vaccines may be needed because they are the only way to protect against difficult pathogens, including chronic infections like malaria and HIV, where the pathogen successfully manipulates the immune system to its advantage, as well as emerging threats, such as pandemic influenza. While attempting to design and license molecular vaccines for these pathogens, significant obstacles have been encountered, such as antigenic polymorphism, the existence of redundant molecular pathways, and host genetic restriction. These obstacles in turn have led to major improvements in molecular technologies. We now have multiple promising platforms to optimize, a variety of excellent models for testing, and burgeoning knowledge of innate and acquired immune responses to inform development efforts.

As illustrated in this review, malaria is playing a crucial role in the development of molecular vaccines. Many novel technologies, such as the evaluation of a plasmid encoding human GM-CSF in healthy adults, have first been tested against *Plasmodium*. Malaria has the advantage of providing an ethical human challenge model, presenting vaccine developers with rapid feedback regarding efficacy, thereby accelerating the testing of new vaccine concepts.

Looking to the future, one of the main challenges for vaccine developers is the complexity of malaria proteins as antigens. The best malaria antigens may prove difficult for the immune system

to recognize, either because of diversionary strategies or for other reasons, such as host mimicry (hiding under the umbrella of self-tolerance), or because evolution has favored amino acid sequences that bind poorly to MHC molecules. For example, the parasite exposes immunodominant domains (such as the NANP repeat of *P. falciparum* CSP) that, while inducing partially protective immune responses, also may serve as decoys diverting the immune response away from subdominant responses that are potentially more protective. Moreover, epitopes may have evolved to stimulate regulatory T cell responses, suppressing rather than enhancing immunity. The success of molecular vaccines for malaria will likely require progress in many areas, including the identification of new antigens, the optimization of vaccine platforms able to deliver multiple antigens, the development of new, safer adjuvants and immunostimulants, and the redesign of antigenic targets to improve immunogenicity.

Acknowledgements

We thank Ashley Birkett for assistance in generating the manuscript outline, Doug Brough for his review of the entire manuscript, Sheetij Dutta, Yimin Wu and Shannon Takala for their reviews of the sections describing their laboratory's work on AMA1, David Lanar for his review of the sections on SAPNs, Blaise Genton for his review of the update on viroplasm and Graham Bentley for his review of the legend for Figure 2. We are grateful to Gary Brice, Adrian Hill, Tupur Husain, David Lanar and Annie Mo for their personal communications, to Judith Epstein, Birgitte Giersing, Gregory Mullen and Vasee Moorthy for permission to republish Figure 1, to Edmond Remarque, Bart Faber and Henk van Westbroek for the original figure of the PfAMA1 crystal structure and legend in Figure 2 and to David Lanar for permission to publish the SAPN diagram and murine protection data in Figure 4.

The study protocols for the clinical trials discussed in the manuscript that were performed by the US Navy or US Army were approved by the Naval Medical Research Center or Walter Reed Army Institute of Research Institutional Review Boards, respectively, in compliance with all applicable Federal regulations governing the protection of human subjects. Likewise, the animal experiments reported herein that were conducted by the US Navy or US Army were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. TLR is a military service member and EA is an employee of the U.S. Government. The work of TLR and EA was prepared as part of official government duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties. The work of KL and TLR was supported by work unit number 6000.RAD1.FA0309.

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, the Department of the Army, the Department of Defense, or the U.S. Government.

References

- Casares S, Richie TL. Immune evasion by malaria parasites: a challenge for vaccine development. *Curr Opin Immunol* 2009; 21:321-30.
- Angov E, Hillier CJ, Kincaid RL, Lyon JA. Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. *PLoS One* 2008; 3:e2189.
- Gao F, Li Y, Decker JM, Peyrel FW, Bibollet-Ruche F, Rodenburg CM, et al. Codon usage optimization of HIV type 1 subtype C gag, pol, env, and nef genes: in vitro expression and immune responses in DNA-vaccinated mice. *AIDS Res Hum Retroviruses* 2003; 19:817-23.
- Narum DL, Kumar S, Rogers WO, Fuhrmann SR, Liang H, Oakley M, et al. Codon optimization of gene fragments encoding Plasmodium falciparum merozoite proteins enhances DNA vaccine protein expression and immunogenicity in mice. *Infect Immun* 2001; 69:7250-3.
- Dobano C, Widera G, Rabussay D, Doolan DL. Enhancement of antibody and cellular immune responses to malaria DNA vaccines by in vivo electroporation. *Vaccine* 2007; 25:6635-45.
- Bruder JT, Chen P, Stefaniak ME, Semenova E, Limbach K, Patterson NB, et al. Development of a multi-antigen multi-stage adenovector-based malaria vaccine that induces robust T-cell and antibody responses (Abstract #6, oral presentation). *Am J Trop Med Hyg* 2008; 79:Supplement.
- Gowda DC, Davidson EA. Protein glycosylation in the malaria parasite. *Parasitol Today* 1999; 15:147-52.
- Stowers AW, Chappell LH, Zhang Y, Kennedy MC, Zou L, Lambert L, et al. A recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys from a lethal challenge with Plasmodium falciparum. *Proc Natl Acad Sci USA* 2002; 99:339-44.
- Stowers AW, Ciocce V, Shimp RL, Lawson M, Hui G, Muratova O, et al. Efficacy of two alternate vaccines based on Plasmodium falciparum merozoite surface protein 1 in an Aotus challenge trial. *Infect Immun* 2001; 69:1536-46.
- Stowers AW, Kennedy MC, Keegan BP, Saul A, Long CA, Miller LH. Vaccination of monkeys with recombinant Plasmodium falciparum apical membrane antigen 1 confers protection against blood-stage malaria. *Infection Immun* 2002; 70:6961-7.
- Giersing B, Miura K, Shimp R, Wang J, Zhou H, Orcutt A, et al. posttranslational modification of recombinant Plasmodium falciparum apical membrane antigen 1: impact on functional immune responses to a malaria vaccine candidate. *Infect Immun* 2005; 73:3963-70.
- Scheibhofer S, Chen D, Weiss R, Khan F, Mostböck S, Fegeding K, et al. Removal of the circumsporozoite protein (CSP) glycosylphosphatidylinositol signal sequence from a CSP DNA vaccine enhances induction of CSP-specific Th2 type immune responses and improves protection against malaria infection. *Eur J Immunol* 2001; 31:692-8.
- Bruna-Romero O, Rocha CD, Tsuji M, Gazzinelli RT. Enhanced protective immunity against malaria by vaccination with a recombinant adenovirus encoding the circumsporozoite protein of Plasmodium lacking the GPI-anchoring motif. *Vaccine* 2004; 22:3575-84.
- Amante FH, Crewther PE, Anders RF, Good MF. A cryptic T cell epitope on the apical membrane antigen 1 of Plasmodium chabaudi adami can prime for an anamnestic antibody response: implications for malaria vaccine design. *J Immunol* 1997; 159:5535-44.
- Egan A, Waterfall M, Pinder M, Holder A, Riley E. Characterization of human T- and B-cell epitopes in the C terminus of Plasmodium falciparum merozoite surface protein 1: evidence for poor T-cell recognition of polypeptides with numerous disulfide bonds. *Infect Immun* 1997; 65:3024-31.
- Bharadwaj A, Sharma P, Joshi SK, Singh B, Chauhan VS. Induction of protective immune responses by immunization with linear multi-epitope peptides based on conserved sequences from Plasmodium falciparum antigens. *Infection Immun* 1998; 66:3232-41.
- Rathore D, Nagarkatti R, Jani D, Chattopadhyay R, de la Vega P, Kumar S, et al. An immunologically cryptic epitope of Plasmodium falciparum circumsporozoite protein facilitates liver cell recognition and induces protective antibodies that block liver cell invasion. *J Bio Chem* 2005; 280:20524-9.
- Plebanski M, Lee EA, Hannan CM, Flanagan KL, Gilbert SC, Gravenor MB, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nat Med* 1999; 5:565-71.
- Plebanski M, Flanagan KL, Lee EA, Reece WH, Hart K, Gelder C, et al. Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of Plasmodium falciparum. *Immunity* 1999; 10:651-60.
- Lee EA, Flanagan KL, Minigo G, Reece WH, Bailey R, Pinder M, et al. Dimorphic Plasmodium falciparum merozoite surface protein-1 epitopes turn off memory T cells and interfere with T cell priming. *Eur J Immunol* 2006; 36:1168-78.
- Dobano C, McTague A, Sette A, Hoffman SL, Rogers WO, Doolan DL. Mutating the anchor residues associated with MHC binding inhibits and deviates CD8+ T cell mediated protective immunity against malaria. *Mol Immunol* 2007; 44:2235-48.
- Hardy LL, Wick DA, Webb JR. Conversion of tyrosine to the inflammation-associated analog 3'-nitrotyrosine at either TCR- or MHC-contact positions can profoundly affect recognition of the MHC class I-restricted epitope of lymphocytic choriomeningitis virus glycoprotein 33 by CD8 T cells. *J Immunol* 2008; 180:5956-62.
- Pasternak ND, Dzikiowski R. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite Plasmodium falciparum. *Int J Biochem Cell Biol* 2009; 41:1463-6.
- Lavazec C, Sanyal S, Templeton TJ. Expression switching in the stator and Pfmc-2TM superfamilies in Plasmodium falciparum. *Mol Microbiol* 2007; 64:1621-34.
- Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, et al. In vitro studies with recombinant Plasmodium falciparum apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. *Infect Immun* 2002; 70:6948-60.
- Sagara I, Dicko A, Ellis RD, Fay MP, Diawara SI, Assadou MH, et al. A randomized controlled phase 2 trial of the blood stage AMA1-C1A1hydrogel malaria vaccine in children in Mali. *Vaccine* 2009; 27:3090-8.
- Dutta S, Lee SY, Batchelor AH, Lanar DE. Structural basis of antigenic escape of a malaria vaccine candidate. *Proc Natl Acad Sci USA* 2007; 104:12488-93.
- Takala SL, Plowe CV. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'. *Parasite Immunol* 2009; 31:560-73.
- Duan J, Mu J, Thera MA, Joy D, Kosakovsky Pond SL, et al. Population structure of the genes encoding the polymorphic Plasmodium falciparum apical membrane antigen 1: implications for vaccine design. *Proc Natl Acad Sci USA* 2008; 105:7857-62.
- Remarque EJ, Faber BW, Kocken CH, Thomas AW. A diversity-covering approach to immunization with Plasmodium falciparum apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits. *Infect Immun* 2008; 76:2660-70.
- Coley AM, Gupta A, Murphy VJ, Bai T, Kim H, Foley M, et al. Structure of the malaria antigen AMA1 in complex with a growth-inhibitory antibody. *PLoS Pathog* 2007; 3:1308-19.
- Collins CA, De Maziere A, van Dijk S, Carlsson F, Klumperman J, Brown EJ. Atg5-independent sequestration of ubiquitinated mycobacteria. *PLoS Pathog* 2009; 5:e1000430.
- Decker JM, Bibollet-Ruche F, Wei X, Wang S, Levy DN, Wang W, et al. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. *J Exp Med* 2005; 201:1407-19.
- Doolan DL, Hoffman SL, Southwood S, Wenworth PA, Sidney J, Chesnut RW, et al. Degenerate cytotoxic T cell epitopes from P. falciparum restricted by multiple HLA-A and HLA-B supertype alleles. *Immunity* 1997; 7:97-112.
- Cai Q, Peng G, Bu L, Lin Y, Zhang L, Lustigmen S, et al. Immunogenicity and in vitro protective efficacy of a polyepitope Plasmodium falciparum candidate vaccine constructed by epitope shuffling. *Vaccine* 2007; 25:5155-65.
- Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, Doolan DL, et al. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis* 2002; 185:1155-64.
- Richie TL, Saul A. Progress and challenges for malaria vaccines. *Nature* 2002; 415:694-701.
- Doolan DL, Southwood S, Chesnut RA, Gomez E, Richards A, Higashimoto YI, et al. HLA-DR-promiscuous T cell epitopes from Plasmodium falciparum pre-erythrocytic-stage antigens restricted by multiple HLA class II alleles. *J Immunol* 2000; 165:1123-37.
- Wizel B, Houghton RA, Parker KC, Coligan JE, Church P, Gordon DM, et al. Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the Plasmodium falciparum sporozoite surface protein 2. *J Exp Med* 1995; 182:1435-45.
- Wizel B, Houghton R, Church P, Tine JA, Lanar DE, Gordon DM, et al. HLA-A2-restricted cytotoxic T lymphocyte responses to multiple Plasmodium falciparum sporozoite surface protein 2 epitopes in sporozoite-immunized volunteers. *J Immunol* 1995; 155:766-75.
- Kumar KA, Sano G, Boscardin S, Nussenzweig RS, Nussenzweig MC, Zavala F, et al. The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. *Nature* 2006; 444:937-40.
- Doolan DL, Hoffman SL. Multi-gene vaccination against malaria: a multi-stage, multi-immune response approach. *Parasitol Today* 1997; 13:171-8.
- Doolan DL, Sedegah M, Hedstrom RC, Hobart P, Charoenvit Y, Hoffman SL. Circumventing genetic restriction of protection against malaria with multi-gene DNA immunization: CD8+ cell-, interferon gamma-, and nitric oxide-dependent immunity. *J Exp Med* 1996; 183:1739-46.
- Weiss WR, Kumar A, Jiang G, Williams J, Bostick A, Conteh S, et al. Protection of rhesus monkeys by a DNA prime/poxvirus boost malaria vaccine depends on optimal DNA priming and inclusion of blood stage antigens. *PLoS One* 2007; 2:e1063.
- Jiang G, Shi M, Conteh S, Richie N, Banania G, Geneshan H, et al. Sterile protection against Plasmodium knowlesi in rhesus monkeys from a malaria vaccine: comparison of heterologous prime boost strategies. *PLoS One* 2009; 4:e6559.
- Rogers WO, Weiss WR, Kumar A, Aguiar JC, Tine JA, Gwadz R, et al. Protection of rhesus macaques against lethal Plasmodium knowlesi malaria by a heterologous DNA priming and poxvirus boosting immunization regimen. *Infect Immun* 2002; 70:4329-35.
- Sedegah M, Charoenvit Y, Minh L, Belmonte M, Majam VF, Abot S, et al. Reduced immunogenicity of DNA vaccine plasmids in mixtures. *Gene Ther* 2004; 11:448-56.

48. Bacon DJ, Sedegah M. Reduced production of RNA transcripts from individual DNA plasmids given in a multivalent DNA vaccine formula. *Hum Vaccin* 2007; 3:48-53.
49. Prieur E, Gilbert SC, Schneider J, Moore AC, Sheu EG, Goonetilleke N, et al. A *Plasmodium falciparum* candidate vaccine based on a six-antigen polyprotein encoded by recombinant poxviruses. *Proc Natl Acad Sci USA* 2004; 101:290-5.
50. Zhou Z, Todd CW, Wohlhueter RM, Price A, Xiao L, Schnake P, et al. Development, characterization and immunogenicity of a multi-stage, multi-valent *Plasmodium falciparum* vaccine antigen (FALVAC-1A) expressed in *Escherichia coli*. *Hum Vaccin* 2006; 2:14-23.
51. Garçon N, Chomez P, Van Mechelen M. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. *Expert Rev Vaccines* 2007; 6:723-39.
52. Heppner DG, Jr., Kester KE, Ockenhouse CF, Tornieporth N, Ofori O, Lyon JA, et al. Towards an RTS,S-based, multi-stage, multi-antigen vaccine against *falciparum* malaria: progress at the Walter Reed Army Institute of Research. *Vaccine* 2005; 23:2243-50.
53. Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. *Pharm Biotechnol* 1995; 6:495-524.
54. Stewart VA, McGrath SM, Walsh DS, Davis S, Hess AS, Ware LA, et al. Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A. *Vaccine* 2006; 24:6483-92.
55. Lawrence G, Cheng QQ, Reed C, Taylor D, Stowers A, Cloonan N, et al. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. *Vaccine* 2000; 18:1925-31.
56. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, et al. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *J Infect Dis* 2002; 185:820-7.
57. Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pf25 and Pvs25 formulated with montanide ISA 51. *PLoS One* 2008; 3:e2636.
58. Roestenberg M, Remarque E, de Jonge E, Hermesen R, Blythman H, Leroy O, et al. Safety and immunogenicity of a recombinant *Plasmodium falciparum* AMA1 malaria vaccine adjuvanted with Alhydrogel, Montanide ISA 720 or AS02. *PLoS One* 2008; 3:e3960.
59. Lahiri A, Das P, Chakravorty D. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. *Vaccine* 2008; 26:6777-83.
60. Ishii KJ, Akira S. Toll or toll-free adjuvant path toward the optimal vaccine development. *J Clin Immunol* 2007; 27:363-71.
61. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 2007; 7:179-90.
62. Schiller M, Metzke D, Luger TA, Grabbe S, Gunzer M. Immune response modifiers--mode of action. *Exp Dermatol* 2006; 15:331-41.
63. Albiger B, Dahlberg S, Henriques-Normark B, Normark S. Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. *J Intern Med* 2007; 261:511-28.
64. Bargieri DY, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espindola NM, et al. New malaria vaccine candidates based on the *Plasmodium vivax* Merozoite Surface Protein-1 and the TLR-5 agonist *Salmonella* Typhimurium FljC flagellin. *Vaccine* 2008; 26:6132-42.
65. Othoro C, Johnston D, Lee R, Soverow J, Bystryjn JC, Nardin E. Enhanced immunogenicity of *Plasmodium falciparum* peptide vaccines using a topical adjuvant containing a potent synthetic Toll-like receptor 7 agonist, imiquimod. *Infect Immun* 2009; 77:739-48.
66. Boland G, Beran J, Lievens M, Sasadeusz J, Denticio P, Nothdurft H, et al. Safety and immunogenicity profile of an experimental hepatitis B vaccine adjuvanted with AS04. *Vaccine* 2004; 23:316-20.
67. Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, et al. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. *Vaccine* 2008; 26:552-61.
68. Coban C, Philipp MT, Purcell JE, Keister DB, Okulate M, Martin DS, et al. Induction of *Plasmodium falciparum* transmission-blocking antibodies in nonhuman primates by a combination of DNA and protein immunizations. *Infect Immun* 2004; 72:253-9.
69. Mullen GE, Ellis RD, Miura K, Malkin E, Nolan C, Hay M, et al. Phase 1 trial of AMA1-C1/Alhydrogel plus CPG 7909: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *PLoS One* 2008; 3:e2940.
70. Schneeberger A, Wagner C, Zemmann A, Luhrs P, Kutil R, Goos M, et al. CpG motifs are efficient adjuvants for DNA cancer vaccines. *J Invest Dermatol* 2004; 123:371-9.
71. Klinman DM, Yamshchikov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J Immunol* 1997; 158:3635-9.
72. Tang CK, Pietersz GA. Intracellular detection and immune signaling pathways of DNA vaccines. *Expert Rev Vaccines* 2009; 8:1161-70.
73. Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther* 2003; 10:935-40.
74. Hartman ZC, Kiang A, Everett RS, Serra D, Yang XY, Clay TM, et al. Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo. *J Virol* 2007; 81:1796-812.
75. Zhu J, Huang X, Yang Y. Innate immune response to adenoviral vectors is mediated by both Toll-like receptor-dependent and -independent pathways. *J Virol* 2007; 81:3170-80.
76. Nociari M, Ocheretina O, Schoggins JW, Falck-Pedersen E. Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. *J Virol* 2007; 81:4145-57.
77. Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, et al. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 2008; 452:103-7.
78. Molinier-Frenkel V, Lengagne R, Gaden F, Hong SS, Choppin J, Gahery-Segard H, et al. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. *J Virol* 2002; 76:127-35.
79. Hutchings CL, Gilbert SC, Hill AV, Moore AC. Novel protein and poxvirus-based vaccine combinations for simultaneous induction of humoral and cell-mediated immunity. *J Immunol* 2005; 175:599-606.
80. Hutchings CL, Birkett AJ, Moore AC, Hill AV. Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge. *Infect Immun* 2007; 75:5819-26.
81. Hidmark AS, Nordstrom EK, Dosenovic P, Forsell MN, Liljestrom P, Karlsson Hedestam GB. Humoral responses against coimmunized protein antigen but not against alphavirus-encoded antigens require alpha/beta interferon signaling. *J Virol* 2006; 80:7100-10.
82. Jones TR, Obaldia N, 3rd, Gramzinski RA, Charoenvit Y, Kolodny N, Kitov S, et al. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. *Vaccine* 1999; 17:3065-71.
83. Kumar S, Jones TR, Oakley MS, Zheng H, Kuppusamy SP, Taye A, et al. CpG oligodeoxynucleotide and Montanide ISA 51 adjuvant combination enhanced the protective efficacy of a subunit malaria vaccine. *Infect Immun* 2004; 72:949-57.
84. Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Ichino M, Barnhart K, et al. A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. *J Immunol* 1998; 161:2325-32.
85. Sedegah M, Weiss W, Sacchi JB, Jr., Charoenvit Y, Hedstrom R, Gowda K, et al. Improving protective immunity induced by DNA-based immunization: priming with antigen and GM-CSF-encoding plasmid DNA and boosting with antigen-expressing recombinant poxvirus. *J Immunol* 2000; 164:5905-12.
86. Wang R, Richie TL, Baraceros MF, Rahardjo N, Gay T, Banania JG, et al. Boosting of DNA vaccine-elicited gamma interferon responses in humans by exposure to malaria parasites. *Infect Immun* 2005; 73:2863-72.
87. Lasaro MO, Tatis N, Hensley SE, Whitbeck JC, Lin SW, Rux JJ, et al. Targeting of antigen to the herpesvirus entry mediator augments primary adaptive immune responses. *Nat Med* 2008; 14:205-12.
88. Yu P, Lee Y, Wang Y, Liu X, Auh S, Gajewski T, et al. Targeting the primary tumor to generate CTL for the effective eradication of spontaneous metastases. *J Immunol* 2007; 179:1960-8.
89. Larsen KC, Spencer AJ, Goodman AL, Gilchrist A, Furze J, Rollier CS, et al. Expression of tak1 and tram induces synergistic pro-inflammatory signalling and adjuvants DNA vaccines. *Vaccine* 2009; 27:5589-98.
90. O'Hagan D, Singh M, Ugozzoli M, Wild C, Barnett S, Chen M, et al. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. *J Virol* 2001; 75:9037-43.
91. Selinsky C, Luke C, Wloch M, Geall A, Hermanson G, Kaslow D, et al. DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases. *Hum Vaccin* 2005; 1:16-23.
92. Wloch MK, Smith LR, Boutsabouloy S, Reyes L, Han C, Kehler J, et al. Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects. *J Infect Dis* 2008; 197:1634-42.
93. Jiang G, Charoenvit Y, Moreno A, Baraceros MF, Banania G, Richie N, et al. Induction of multi-antigen multi-stage immune responses against *Plasmodium falciparum* in rhesus monkeys, in the absence of antigen interference, with heterologous DNA prime/poxvirus boost immunization. *Malar J* 2007; 6:135.
94. Sedegah M, Rogers WO, Belmonte A, Belmonte M, Banania G, Patterson N, et al. Vaxfectin enhances immunogenicity and protective efficacy of *P. yoelii* circumsporozoite DNA vaccines. *Vaccine* 2006; 24:1921-7.
95. Brice GT. Vaxfectin® enhances antibody and T-cell responses of malaria plasmid DNA vaccines administered at low doses in nonhuman primates (Abstract). 58th Annual Meeting of the American Society of Tropical Medicine & Hygiene, Washington DC 2009.
96. Ophorst OJ, Radošević K, Ourwhand K, van Beem W, Mintardjo R, Sijsma J, et al. Expression and immunogenicity of the *Plasmodium falciparum* circumsporozoite protein: the role of GPI signal sequence. *Vaccine* 2007; 25:1426-36.
97. Ophorst OJ, Kostense S, Goudsmit J, De Swart RL, Verhaagh S, Zakhartchouk A, et al. An adenoviral type 5 vector carrying a type 35 fiber as a vaccine vehicle: DC targeting, cross neutralization, and immunogenicity. *Vaccine* 2004; 22:3035-44.
98. Nanda A, Lynch D, M., Goudsmit J, Lemckert, A. A., Ewald, B. A., Sumida, S. M., et al. Immunogenicity of recombinant fiber-chimeric adenovirus serotype 35 vector-based vaccines in mice and rhesus monkeys. *J Virol* 2005; 79:14161-8.

99. Cheng C, Gall JG, Kong WP, Sheets RL, Gomez PL, King CR, et al. Mechanism of Ad5 vaccine immunity and toxicity: fiber shaft targeting of dendritic cells. *PLoS Pathog*. 2007 Feb;3(2):e25.
100. Ogun SA, Dumon-Seignovet L, Marchand JB, Holder AA, Hill F. The oligomerization domain of C4-binding protein (C4bp) acts as an adjuvant, and the fusion protein comprised of the 19-kilodalton merozoite surface protein 1 fused with the murine C4bp domain protects mice against malaria. *Infect Immun* 2008; 76:3817-23.
101. Draper SJ, Moore AC, Goodman AL, Long CA, Holder AA, Gilbert SC, et al. Effective induction of high-titer antibodies by viral vector vaccines. *Nat Med* 2008; 14:819-21.
102. Qian F, Wu Y, Muratova O, Zhou H, Dobrescu G, Duggan P, et al. Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. *Vaccine* 2007; 25:3923-33.
103. Applequist SE, Rollman E, Wareing MD, Liden M, Rozell B, Hinkula J, et al. Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin. *J Immunol* 2005; 175:3882-91.
104. Tosch C, Geist M, Ledoux C, Ziller-Remi C, Paul S, Erbs P, et al. Adenovirus-mediated gene transfer of pathogen-associated molecular patterns for cancer immunotherapy. *Cancer Gene Ther* 2008; 16:310-9.
105. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 1996; 271:348-50.
106. Ross TM, Xu Y, Bright RA, Robinson HL. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. *Nat Immunol* 2000; 1:127-31.
107. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dworki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993; 259:1745-9.
108. Sedegah M, Hedstrom R, Hobart P, Hoffman SL. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc Natl Acad Sci USA* 1994; 91:9866-70.
109. MacGregor RR, Boyer JD, Ugen KE, Lacy KE, Gluckman SJ, Bagarazzi ML, et al. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* 1998; 178:92-100.
110. Tacket CO, Roy MJ, Widera G, Swain WF, Broome S, Edelman R. Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* 1999; 17:2826-9.
111. Le TP, Coonan KM, Hedstrom RC, Charoenvit Y, Sedegah M, Epstein JE, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* 2000; 18:1893-901.
112. Epstein JE, Gorak EJ, Charoenvit Y, Wang R, Freydenberg N, Osinowo O, et al. Safety, tolerability, and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes. *Hum Gene Ther* 2002; 13:1551-60.
113. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998; 282:476-80.
114. Wang J, Chun T, Lo JC, Wu Q, Wang Y, Foster A, et al. The critical role of LIGHT, a TNF family member, in T cell development. *J Immunol* 2001; 167:5099-105.
115. Garver KA, LaPatra SE, Kurath G. Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook *Oncorhynchus tshawytscha* and sockeye *O. nerka* salmon. *Dis Aquat Organ* 2005; 64:13-22.
116. Bergman PJ, Camps-Palau MA, McKnight JA, Leibman NF, Craft DM, Leung C, et al. Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. *Vaccine* 2006; 24:4582-5.
117. Thacker EL, Holtkamp DJ, Khan AS, Brown PA, Draghia-Akli R. Plasmid-mediated growth hormone-releasing hormone efficacy in reducing disease associated with *Mycoplasma hyopneumoniae* and porcine reproductive and respiratory syndrome virus infection. *J Anim Sci* 2006; 84:733-42.
118. Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, et al. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. *Proc Natl Acad Sci USA* 2001; 98:10817-22.
119. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci USA* 1993; 90:11478-82.
120. Jones S, Evans K, McElwaine-John H, Sharpe M, Oxford J, Lambkin-Williams R, et al. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. *Vaccine* 2009; 27:2506-12.
121. Low L, Mander A, McCann K, Dearnaley D, Tjelle T, Mathiesen I, et al. DNA Vaccination with Electroporation Induces Increased Antibody Responses in Patients with Prostate Cancer. *Hum Gene Ther* 2009; 20:1269-78.
122. Geisbert TW, Daddario-Dicaprio KM, Lewis MG, Geisbert JB, Grolla A, Leung A, et al. Vesicular stomatitis virus-based ebola vaccine is well-tolerated and protects immunocompromised nonhuman primates. *PLoS Pathog* 2008; 4:e1000225.
123. Tao D, Barba-Spaeth G, Rai U, Nussenzweig V, Rice CM, Nussenzweig RS. Yellow fever 17D as a vaccine vector for microbial CTL epitopes: protection in a rodent malaria model. *J Exp Med* 2005; 201:201-9.
124. Bonaldo MC, Garratt RC, Caufour PS, Freire MS, Rodrigues MM, Nussenzweig RS, et al. Surface expression of an immunodominant malaria protein B cell epitope by yellow fever virus. *J Mol Biol* 2002; 315:873-85.
125. Lanar DE, Tine JA, de Taisne C, Seguin MC, Cox WI, Winslow JP, et al. Attenuated vaccinia virus-circumsporozoite protein recombinants confer protection against rodent malaria. *Infect Immun* 1996; 64:1666-71.
126. Tine JA, Lanar DE, Smith DM, Welde BT, Schultheiss P, Ware LA, et al. NYVAC-Pf7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *Infect Immun* 1996; 64:3833-44.
127. Ockenhouse CF, Sun PF, Lanar DE, Welde BT, Hall BT, Kester K, et al. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis* 1998; 177:1664-73.
128. Dunachie SJ, Hill AV. Prime-boost strategies for malaria vaccine development. *J Exp Biol* 2003; 206:3771-9.
129. Gilbert SC, Plebanski M, Harris SJ, Allsopp CE, Thomas R, Layton GT, et al. A protein particle vaccine containing multiple malaria epitopes. *Nat Biotechnol* 1997; 15:1280-4.
130. Dunachie SJ, Walther M, Epstein JE, Keating S, Berthoud T, Andrews L, et al. A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naïve adults against *Plasmodium falciparum* sporozoite challenge. *Infect Immun* 2006; 74:5933-42.
131. McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* 2003; 9:729-35.
132. Webster DP, Dunachie S, Vuola JM, Berthoud T, Keating S, Laidlaw SM, et al. Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc Natl Acad Sci USA* 2005; 102:4836-41.
133. Bejon P, Andrews L, Andersen RF, Dunachie S, Webster D, Walther M, et al. Calculation of liver-to-blood inocula, parasite growth rates, and preerythrocytic vaccine efficacy, from serial quantitative polymerase chain reaction studies of volunteers challenged with malaria sporozoites. *J Infect Dis* 2005; 191:619-26.
134. Moorthy VS, Imoukhuede EB, Keating S, Pinder M, Webster D, Skinner MA, et al. Phase I evaluation of 3 highly immunogenic prime-boost regimens, including a 12-month reboosting vaccination, for malaria vaccination in Gambian men. *J Infect Dis* 2004; 189:2213-9.
135. Bejon P, Mwacharo J, Kai O, Mwangi T, Milligan P, Todryk S, et al. A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS Clin Trials*. 2006; 1:e29.
136. Jacobs SC, Stephenson JR, Wilkinson GW. High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: protection elicited in a murine model. *J Virol* 1992; 66:2086-95.
137. Xiang ZQ, Yang Y, Wilson JM, Ertl HC. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* 1996; 219:220-7.
138. Lubeck MD, Natuk R, Myagikhik M, Kalyan N, Aldrich K, Sinangil F, et al. Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunization. *Nat Med* 1997; 3:651-8.
139. Rodrigues EG, Zavala F, Eichinger D, Wilson JM, Tsuji M. Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J Immunol* 1997; 158:1268-74.
140. Sullivan NJ, Geisbert TW, Geisbert JB, Xu L, Yang ZY, Roederer M, et al. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 2003; 424:681-4.
141. Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002; 415:331-5.
142. Gilbert SC, Schneider J, Hannan CM, Hu JT, Plebanski M, Sinden R, et al. Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimens. *Vaccine* 2002; 20:1039-45.
143. Tan Y, Hackett NR, Boyer JL, Crystal RG. Protective immunity evoked against anthrax lethal toxin after a single intramuscular administration of an adenovirus-based vaccine encoding humanized protective antigen. *Hum Gene Ther* 2003; 14:1673-82.

144. Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, et al. Single mucosal, but not parenteral, immunization with recombinant adenovirus-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* 2004; 173:6357-65.
145. Epstein SL, Kong WP, Misplon JA, Lo CY, Tumpey TM, Xu L, et al. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* 2005; 23:5404-10.
146. Worgall S, Krause A, Rivara M, Hee KK, Vintayen EV, Hackett NR, et al. Protection against *P. aeruginosa* with an adenovirus vector containing an OprF epitope in the capsid. *J Clin Invest* 2005; 115:1281-9.
147. Ophorst OJ, Radosevic K, Havenga MJ, Pau MG, Holterman L, Berkhout B, et al. Immunogenicity and protection of a recombinant human adenovirus serotype 35-based malaria vaccine against *Plasmodium yoelii* in mice. *Infect Immun* 2006; 74:313-20.
148. Rodrigues EG, Claassen J, Lee S, Wilson JM, Nussenzeig RS, Tsuji M. Interferon-gamma-independent CD8⁺ T cell-mediated protective anti-malaria immunity elicited by recombinant adenovirus. *Parasite Immunol* 2000; 22:157-60.
149. Sridhar S, Reyes-Sandoval A, Draper SJ, Moore AC, Gilbert SC, Gao GP, et al. Single-dose protection against *Plasmodium berghei* by a simian adenovirus vector using a human cytomegalovirus promoter containing intron A. *J Virol* 2008; 82:3822-33.
150. Catanzaro AT, Koup RA, Roederer M, Bailer RT, Enama ME, Moodie Z, et al. Phase 1 safety and immunogenicity evaluation of a multiclade HIV-1 candidate vaccine delivered by a replication-defective recombinant adenovirus vector. *J Infect Dis* 2006; 194:1638-49.
151. Bruder JT, Kovsed I. Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression. *J Virol* 1997; 71:398-404.
152. Wang H, Lieber A. A helper-dependent capsid-modified adenovirus vector expressing adenovirus-associated virus rep78 mediates site-specific integration of a 27-kilobase transgene cassette. *J Virol* 2006; 80:11699-709.
153. Raja NU, Holman DH, Wang D, Raviprakash K, Juompan LY, Deitz SB, et al. Induction of bivalent immune responses by expression of dengue virus type 1 and type 2 antigens from a single complex adenoviral vector. *Am J Trop Med Hyg* 2007; 76:743-51.
154. Vogels R, Zuidgeest D, van Meerendonk M, Compansen A, Gillissen G, Sijtsma J, et al. High-level expression from two independent expression cassettes in replication-incompetent adenovirus type 35 vector. *J Gen Virol* 2007; 88:2915-24.
155. Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovsed I. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. *J Virol* 1996; 70:6497-501.
156. Koup RA, Lamoreaux L, Zarkowsky D, Bailer RT, King CR, Gall JG, et al. Replication-defective adenovirus vectors with multiple deletions do not induce measurable vector-specific T cells in human trials. *J Virol* 2009; 83:6318-22.
157. Vogels R, Zuidgeest D, van Rijnsoever R, Hartkoorn E, Damen I, de Bethune M-P, et al. Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* 2003; 77:8263-71.
158. Thorne AR, Vogels R, Kaspers J, Weverling GJ, Holterman L, Lemckert AA, et al. Age dependence of adenovirus-specific neutralizing antibody titers in individuals from sub-Saharan Africa. *J Clin Microbiol* 2006; 44:3781-3.
159. Kostense S, Koudstaal W, Sprangers M, Weverling GJ, Penders G, Helmus N, et al. Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. *AIDS* 2004; 18:1213-6.
160. Sumida SM, Truitt DM, Lemckert AA, Vogels R, Custers JH, Addo MM, et al. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol* 2005; 174:179-85.
161. Lemckert AA, Sumida SM, Holterman L, Vogels R, Truitt DM, Lynch DM, et al. Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* 2005; 79:9694-701.
162. Casimiro DR, Chen L, Fu TM, Evans RK, Caulfield MJ, Davies ME, et al. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol* 2003; 77:6305-13.
163. Hierholzer JC, Stone YO, Broderman JR. Antigenic relationships among the 47 human adenoviruses determined in reference horse antisera. *Arch Virol* 1991; 121:179-97.
164. Barouch DH, Pau MG, Custers JH, Koudstaal W, Kostense S, Havenga MJ, et al. Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* 2004; 172:6290-7.
165. Farina SF, Gao GP, Xiang ZQ, Rux JJ, Burnett RM, Alvira MR, et al. Replication-defective vector based on a chimpanzee adenovirus. *J Virol* 2001; 75:11603-13.
166. Fitzgerald JC, Gao GP, Reyes-Sandoval A, Pavlakis GN, Xiang ZQ, Wlazlo AP, et al. A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J Immunol* 2003; 170:1416-22.
167. Pinto AR, Fitzgerald JC, Giles-Davis W, Gao GP, Wilson JM, Ertl HC. Induction of CD8⁺ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* 2003; 171:6774-9.
168. Xiang Z, Gao G, Reyes-Sandoval A, Cohen CJ, Li Y, Bergelson JM, et al. Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product. *J Virol* 2002; 76:2667-75.
169. McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, James H, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 2008; 372:1894-905.
170. Priddy FH, Brown D, Kublin J, Monahan K, Wright DP, Lalezari J, et al. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. *Clin Infect Dis* 2008; 46:1769-81.
171. Shiver JW, Emini EA. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med* 2004; 55:355-72.
172. Hensley SE, Cun AS, Giles-Davis W, Li Y, Xiang Z, Lasaro MO, et al. Type I interferon inhibits antibody responses induced by a chimpanzee adenovirus vector. *Mol Ther* 2007; 15:393-403.
173. Gall J, Kass-Eisler A, Leinwand L, Falck-Pedersen E. Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J Virol* 1996; 70:2116-23.
174. Gall JG, Crystal RG, Falck-Pedersen E. Construction and characterization of hexon-chimeric adenoviruses: specification of adenovirus serotype. *J Virol* 1998; 72:10260-4.
175. Roy S, Shirley PS, McClelland A, Kaleko M. Circumvention of immunity to the adenovirus major coat protein hexon. *J Virol* 1998; 72:6875-9.
176. Roberts DM, Nanda A, Havenga MJ, Abbink P, Lynch DM, Ewald BA, et al. Hexon-chimeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 2006; 441:239-43.
177. Bruder JT, Ping C, Semenova E, Thomas C, Hamilton M, Patterson NB, et al. Advanced generation adenovirus-based vectors for malaria vaccine development (Abstract #644, poster presentation). *Am J Trop Med Hyg* 2008; 79:Supplement.
178. Worgall S, Busch A, Rivara M, Bonnyay D, Leopold PL, Merritt R, et al. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. *J Virol* 2004; 78:2572-80.
179. Krause A, Joh JH, Hackett NR, Roelvink PW, Bruder JT, Wickham TJ, et al. Epitopes expressed in different adenovirus capsid proteins induce different levels of epitope-specific immunity. *J Virol* 2006; 80:5523-30.
180. Worgall S, Krause A, Qiu J, Joh J, Hackett NR, Crystal RG. Protective immunity to *Pseudomonas aeruginosa* induced with a capsid-modified adenovirus expressing *P. aeruginosa* OprF. *J Virol* 2007; 81:13801-8.
181. Dietrich G, Spreng S, Favre D, Viret JF, Guzman CA. Live attenuated bacteria as vectors to deliver plasmid DNA vaccines. *Curr Opin Mol Ther* 2003; 5:10-9.
182. Schafer R, Portnoy DA, Brassell SA, Paterson Y. Induction of a cellular immune response to a foreign antigen by a recombinant *Listeria monocytogenes* vaccine. *J Immunol* 1992; 149:53-9.
183. Rayevskaya MV, Frankel FR. Systemic immunity and mucosal immunity are induced against human immunodeficiency virus Gag protein in mice by a new hyperattenuated strain of *Listeria monocytogenes*. *J Virol* 2001; 75:2786-91.
184. Gonzalez C, Hone D, Noriega FR, Tacket CO, Davis JR, Losonsky G, et al. *Salmonella typhi* vaccine strain CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*: strain construction and safety and immunogenicity in humans. *J Infect Dis* 1994; 169:927-31.
185. Wu S, Beier M, Szein MB, Galen J, Pickett T, Holder AA, et al. Construction and immunogenicity in mice of attenuated *Salmonella typhi* expressing *Plasmodium falciparum* merozoite surface protein 1 (MSP-1) fused to tetanus toxin fragment C. *J Biotechnol* 2000; 83:125-35.
186. Chinchilla M, Pasetti MF, Medina-Moreno S, Wang JY, Gomez-Duarte OG, Stout R, et al. Enhanced immunity to *Plasmodium falciparum* circumsporozoite protein (PfCSP) by using *Salmonella enterica* serovar Typhi expressing PfCSP and a PfCSP-encoding DNA vaccine in a heterologous prime-boost strategy. *Infect Immun* 2007; 75:3769-79.
187. McKelvie ND, Khan SA, Karavolos MH, Bulmer DM, Lee JJ, DeMarco R, et al. Genetic detoxification of an aroA *Salmonella enterica* serovar Typhimurium vaccine strain does not compromise protection against virulent *Salmonella* and enhances the immune responses towards a protective malarial antigen. *FEMS Immunol Med Microbiol* 2008; 52:237-46.
188. Rapeah S, Norazmi MN. Immunogenicity of a recombinant *Mycobacterium bovis* bacille Calmette-Guerin expressing malarial and tuberculosis epitopes. *Vaccine* 2006; 24:3646-53.
189. Beveridge NE, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, et al. Immunisation with BCG and recombinant MVA85A induces long-lasting, polyfunctional *Mycobacterium tuberculosis*-specific CD4⁺ memory T lymphocyte populations. *Eur J Immunol* 2007; 37:3089-100.
190. Kwon MN. Shigellosis: the current status of vaccine development. *Curr Opin Infect Dis* 2008; 21:313-8.
191. Moorthy SA, Yasawardena SG, Ramasamy R. Age-dependent systemic antibody responses and immunisation-associated changes in mice orally and nasally immunised with *Lactococcus lactis* expressing a malaria parasite protein. *Vaccine* 2009; 27:4947-52.

192. Charest H, Sedegah M, Yap GS, Gazzinelli RT, Caspar P, Hoffman SL, et al. Recombinant attenuated *Toxoplasma gondii* expressing the *Plasmodium yoelii* circumsporozoite protein provides highly effective priming for CD8+ T cell-dependent protective immunity against malaria. *J Immunol* 2000; 165:2084-92.
193. Chackerian B. Virus-like particles: flexible platforms for vaccine development. *Expert Rev Vaccines* 2007; 6:381-90.
194. Grgacic EV, Anderson DA. Virus-like particles: passport to immune recognition. *Methods* 2006; 40:60-5.
195. van Oers MM. Vaccines for viral and parasitic diseases produced with baculovirus vectors. *Adv Virus Res* 2006; 68:193-253.
196. Li TC, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, et al. Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* 2005; 79:12999-3006.
197. Pillay S, Meyers A, Williamson AL, Rybicki EP. Optimization of chimeric HIV-1 virus-like particle production in a baculovirus-insect cell expression system. *Biotechnol Prog* 2009; 25:1153-60.
198. Manayani DJ, Thomas D, Dryden KA, Reddy V, Siladi ME, Marlett JM, et al. A viral nanoparticle with dual function as an anthrax antitoxin and vaccine. *PLoS Pathog* 2007; 3:1422-31.
199. Schodel F, Peterson D, Milich DR, Charoenvit Y, Sadoff J, Wirtz R. Immunization with hybrid hepatitis B virus core particles carrying circumsporozoite antigen epitopes protects mice against *Plasmodium yoelii* challenge. *Behring Inst Mitt* 1997;114-9.
200. Gregson AL, Oliveira G, Ortho C, Calvo-Calle JM, Thornton GB. Phase I Trial of an Alhydrogel Adjuvanted Hepatitis B Core Virus-Like Particle Containing Epitopes of *Plasmodium falciparum* Circumsporozoite Protein. *PLoS One* 2008; 3:e1556.
201. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children: randomised controlled trial. *Lancet* 2004; 364:1411-20.
202. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Aide P, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 2005; 366:2012-8.
203. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Morris P, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naïve adults: safety, efficacy, and immunologic associates of protection. *J Infect Dis* 2009; 200:337-46.
204. Moser C, Amacker M, Kammer AR, Rasi S, Westerfeld N, Zurbriggen R. Influenza viroosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations. *Expert Rev Vaccines* 2007; 6:711-21.
205. Mueller MS, Renard A, Boato F, Vogel D, Naegeli M, Zurbriggen R, et al. Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of *Plasmodium falciparum* apical membrane antigen 1. *Infect Immun* 2003; 71:4749-58.
206. Genton B, Pluschke G, Degen L, Kammer AR, Westerfeld N, Okitsu SL, et al. A randomized placebo-controlled phase Ia malaria vaccine trial of two virosome-formulated synthetic peptides in healthy adult volunteers. *PLoS One* 2007; 2:e1018.
207. Thompson FM, Porter DW, Okitsu SL, Westerfeld N, Vogel D, Todryk S, et al. Evidence of blood stage efficacy with a virosomal malaria vaccine in a phase IIa clinical trial. *PLoS One* 2008; 3:e1493.
208. Kaba SA, Brando C, Guo Q, Mittelholzer C, Senthikumar R, Tropel D, et al. A non-adjuvanted polypeptide nanoparticle vaccine confers long-lasting protection against rodent malaria. *J Immunol* 2009; 183:7268-77.
209. Tamminga C, Sedegah M, Chuang I, Regis D, Mendoza-Silveiras J, Steinbeiss V, et al. Safety, tolerability, immunogenicity, and protective efficacy of a multi-stage, multi-antigen adenovirus-vectored *P. falciparum* malaria vaccine, in healthy, malaria-naïve adults (Abstract) Twelfth Annual Conference on Vaccine Research 2009.
210. Krieg AM, Efler SM, Wittpoth M, Al Adhami MJ, Davis HL. Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J Immunother* 2004; 27:460-71.
211. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008; 372:1881-93.
212. Bork P, Rohde K. More von Willebrand factor type A domains? Sequence similarities with malaria thrombospondin-related anonymous protein, dihydropyridine-sensitive calcium channel and inter-alpha-trypsin inhibitor. *Biochem J* 1991; 279(Pt 3):908-10.
213. Chattopadhyay R, Rathore D, Fujioka H, Kumar S, de la Vega P, Haynes D, et al. PfSPATR, a *Plasmodium falciparum* protein containing an altered thrombospondin type I repeat domain is expressed at several stages of the parasite life cycle and is the target of inhibitory antibodies. *J Biol Chem* 2003; 278:25977-81.
214. Wood AJ, Darbyshire J. Injury to research volunteers - the clinical-research nightmare. *N Engl J Med* 2006; 354:1869-71.
215. Bejon P, Peshu N, Gilbert SC, Lowe BS, Molyneux CS, Forsdyke J, et al. Safety profile of the viral vectors of attenuated fowlpox strain FP9 and modified vaccinia virus Ankara recombinant for either of 2 preerythrocytic malaria antigens, ME-TRAP or the circumsporozoite protein, in children and adults in Kenya. *Clin Infect Dis* 2006; 42:1102-10.
216. Imoukhuede EB, Berthoud T, Milligan P, Bojang K, Ismaili J, Keating S, et al. Safety and immunogenicity of the malaria candidate vaccines FP9 CS and MVA CS in adult Gambian men. *Vaccine* 2006; 24:6526-33.
217. Walther M, Thompson FM, Dunachie S, Keating S, Todryk S, Berthoud T, et al. Safety, immunogenicity, and efficacy of prime-boost immunization with recombinant poxvirus FP9 and modified vaccinia virus Ankara encoding the full-length *Plasmodium falciparum* circumsporozoite protein. *Infect Immun* 2006; 74:2706-16.
218. Webster DP, Dunachie S, McConkey S, Poulton I, Moore AC, Walther M, et al. Safety of recombinant fowlpox strain FP9 and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers. *Vaccine* 2006; 24:3026-34.
219. Van Kampen KR, Shi Z, Gao P, Zhang J, Foster KW, Chen DT, et al. Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine* 2005; 23:1029-36.
220. Harro CD, Robertson MN, Lally MA, O'Neill LD, Edupuganti S, Goepfert PA, et al. Safety and immunogenicity of adenovirus-vectored near-consensus HIV type 1 clade B gag vaccines in healthy adults. *AIDS Res Hum Retroviruses* 2009; 25:103-14.
221. Harro C, Sun X, Stek JE, Leavitt RY, Mehrotra DV, Wang F, et al. Safety and immunogenicity of the Merck adenovirus serotype 5 (MRKAd5) and MRKAd6 human immunodeficiency virus type 1 trigenic vaccines alone and in combination in healthy adults. *Clin Vaccine Immunol* 2009; 16:1285-92.
222. Moorthy VS, McConkey S, Roberts M, Gothard P, Arulanantham N, Degano P, et al. Safety of DNA and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers. *Vaccine* 2003; 21:1995-2002.
223. Moorthy VS, Pinder M, Reece WH, Watkins K, Atabani S, Hannan C, et al. Safety and immunogenicity of DNA/modified vaccinia virus Ankara malaria vaccination in African adults. *J Infect Dis* 2003; 188:1239-44.
224. Epstein JE, Charoenvit Y, Kester KE, Wang R, Newcomer R, Fitzpatrick S, et al. Safety, tolerability, and antibody responses in humans after sequential immunization with a PfCSP DNA vaccine followed by the recombinant protein vaccine RTS,S/AS02A. *Vaccine* 2004; 22:1592-603.
225. Nardin EH, Oliveira GA, Calvo-Calle JM, Wetzel K, Maier C, Birkett AJ, et al. Phase I testing of a malaria vaccine composed of hepatitis B virus core particles expressing *Plasmodium falciparum* circumsporozoite epitopes. *Infect Immun* 2004; 72:6519-27.
226. Oliveira GA, Wetzel K, Calvo-Calle JM, Nussenzweig R, Schmidt A, Birkett A, et al. Safety and enhanced immunogenicity of a hepatitis B core particle *Plasmodium falciparum* malaria vaccine formulated in adjuvant Montanide ISA 720 in a phase I trial. *Infect Immun* 2005; 73:3587-97.
227. Walther M, Dunachie S, Keating S, Vuola JM, Berthoud T, Schmidt A, et al. Safety, immunogenicity and efficacy of a pre-erythrocytic malaria candidate vaccine, ICC-1132 formulated in Seppic ISA 720. *Vaccine* 2005; 23:857-64.
228. Wang Z, Tfoilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, et al. Detection of integration of plasmid DNA into host genomic DNA following intramuscular infection and electroporation. *Gene Ther* 2004; 11:711-21.
229. Mayr A, Strickl H, Muller HK, Danner K, Singer H. [The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl)]. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene Erste Abteilung Originale* 1978; 167:375-90.
230. Martin T, Parker SE, Hedstrom R, Le T, Hoffman SL, Norman J, et al. Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. *Hum Gene Ther* 1999; 10:759-68.
231. Parker SE, Monteith D, Horton H, Hof R, Hernandez P, Vilalta A, et al. Safety of a GM-CSF adjuvant-plasmid DNA malaria vaccine. *Gene Ther* 2001; 8:1011-23.
232. Sheets RL, Stein J, Manetz TS, Duffy C, Nason M, Andrews C, et al. Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. *Toxicol Sci* 2006; 91:610-9.
233. Sheets RL, Stein J, Manetz TS, Andrews C, Bailer R, Rathmann J, et al. Toxicological safety evaluation of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar despite differing plasmid backbones or gene inserts. *Toxicol Sci* 2006; 91:620-30.
234. Mullen GE, Giersing BK, Ajose-Popoola O, Davis HL, Kothe C, Zhou H, et al. Enhancement of functional antibody responses to AMA1-C1/Alhydrogel, a *Plasmodium falciparum* malaria vaccine, with CpG oligodeoxynucleotide. *Vaccine* 2006; 24:2497-505.
235. Mullen GE, Aebig JA, Dobrescu G, Rausch K, Lambert L, Long CA, et al. Enhanced antibody production in mice to the malaria antigen AMA1 by CPG 7909 requires physical association of CpG and antigen. *Vaccine* 2007; 25:5343-7.

236. Ellis RD, Mullen GE, Pierce M, Martin LB, Miura K, Fay MP, et al. A Phase 1 study of the blood-stage malaria vaccine candidate AMA1-C1/Alhydrogel with CPG 7909, using two different formulations and dosing intervals. *Vaccine* 2009; 27:4104-9.
237. Richie TLea. Safety, Immunogenicity and Efficacy of MuStDO 5, a 5 Gene Sporozoite/Hepatic Stage P. falciparum DNA Vaccine Combined with Human GM-CSF DNA (Abstract). *Am J Trop Med Hyg* 2001 65(3 Suppl):132-478.
238. Bai T, Becker M, Gupta A, Strike P, Murphy VJ, Anders RF, et al. Structure of AMA1 from Plasmodium falciparum reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket. *Proc Natl Acad Sci U S A* 2005; 102:12736-41.
239. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; 18:2714-23.
240. Remarque EJ, Faber BW, Kocken CH, Thomas AW. Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol* 2008; 24:74-84.
241. Miura K, Zhou H, Diouf A, Moretz SE, Fay MP, Miller LH, et al. Anti-apical-membrane-antigen-1 antibody is more effective than anti-42-kilodalton-merozoite-surface-protein-1 antibody in inhibiting plasmodium falciparum growth, as determined by the in vitro growth inhibition assay. *Clin Vaccine Immunol* 2009; 16:963-8.
242. Osier FH, Fegan G, Polley SD, Murungi L, Verra F, Tetteh KK, et al. Breadth and magnitude of antibody responses to multiple Plasmodium falciparum merozoite antigens are associated with protection from clinical malaria. *Infect Immun* 2008; 76:2240-8.
243. Iriemenam NC, Khijelsied AH, Nasir A, ElGhazali G, Giha HA, Elhassan AETM, et al. Antibody responses to a panel of Plasmodium falciparum malaria blood-stage antigens in relation to clinical disease outcome in Sudan. *Vaccine* 2009; 27:62-71.
244. Stanisic DI, Richards JS, McCallum FJ, Michon P, King CL, Schoepflin S, et al. Immunoglobulin G subclass-specific responses against Plasmodium falciparum merozoite antigens are associated with control of parasitemia and protection from symptomatic illness. *Infect Immun* 2009; 77:1165-74.
245. Silvie O, Franetich JF, Charrin S, Mueller MS, Siau A, Bodescot M, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by Plasmodium falciparum sporozoites. *J Biol Chem* 2004; 279:9490-6.
246. Narum DL, Thomas AW. Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. *Mol Biochem Parasitol* 1994; 67:59-68.
247. Howell SA, Well I, Fleck SL, Kettleborough C, Collins CR, Blackman MJ. A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage. *J Biol Chem* 2003; 278:23890-8.
248. Dutta S, Haynes JD, Moch JK, Barbosa A, Lanar DE. Invasion-inhibitory antibodies inhibit proteolytic processing of apical membrane antigen 1 of Plasmodium falciparum merozoites. *Proc Natl Acad Sci USA* 2003; 100:12295-300.
249. Triglia T, Healer J, Caruana SR, Hodder AN, Anders RF, Crabb BS, et al. Apical membrane antigen 1 plays a central role in erythrocyte invasion by Plasmodium species. *Mol Microbiol* 2000; 38:706-18.
250. Pizarro JC, Vulliez-Le Normand B, Chesne-Seck ML, Collins CR, Withers-Martinez C, Hackett F, et al. Crystal structure of the malaria vaccine candidate apical membrane antigen 1. *Science* 2005; 308:408-11.
251. Chesne-Seck ML, Pizarro JC, Vulliez-Le Normand B, Collins CR, Blackman MJ, Faber BW, et al. Structural comparison of apical membrane antigen 1 orthologues and paralogues in apicomplexan parasites. *Mol Biochem Parasitol* 2005; 144:55-67.
252. The European Medicine Agency—Report from the CPMG Gene Therapy Expert Group Meeting, 26th-27th February, 2004/ 2004.
253. Harui A, Suzuki S, Kochanek S, Mitani K. Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol* 1999; 73:6141-6.
254. Mitani K, Kubo S. Adenovirus as an integrating vector. *Curr Gene Ther* 2002; 2:135-44.
255. Kuwano K, Kawasaki M, Kunitake R, Hagimoto N, Nomoto Y, Matsuba T, et al. Detection of group C adenovirus DNA in small-cell lung cancer with the nested polymerase chain reaction. *J Cancer Res Clin Oncol* 1997; 123:377-82.
256. Fernandez-Soria V, Bornstein R, Forteza J, Parada C, Sanchez-Prieto R, Ramon y Cajal S. Inconclusive presence of adenovirus sequences in human leukemias and lymphomas. *Oncol Rep* 2002; 9:897-902.
257. Garnett CT, Erdman D, Xu W, Gooding LR. Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. *J Virol* 2002; 76:10608-16.
258. Mahr JA, Boss JM, Gooding LR. The adenovirus $e3$ promoter is sensitive to activation signals in human T cells. *J Virol* 2003; 77:1112-9.
259. Kosulin K, Haberler C, Hainfellner JA, Amann G, Lang S, Lion T. Investigation of adenovirus occurrence in pediatric tumor entities. *J Virol* 2007; 81:7629-35.
260. Hall SJ, Bar-Chama N, Ta S, Gordon JW. Direct exposure of mouse spermatogenic cells to high doses of adenovirus gene therapy vector does not result in germ cell transduction. *Hum Gene Ther* 2000; 11:1705-12.
261. Gordon JW. Direct exposure of mouse ovaries and oocytes to high doses of an adenovirus gene therapy vector fails to lead to germ cell transduction. *Mol Ther* 2001; 3:557-64.
262. Shimwell NJ, Martin A, Bruton RK, Blackford AN, Sedgwick GG, Gallimore PH, et al. Adenovirus 5 E1A is responsible for increased expression of insulin receptor substrate 4 in established adenovirus 5-transformed cell lines and interacts with IRS components activating the PI3 kinase/Akt signalling pathway. *Oncogene* 2009; 28:686-97.
263. Strath J, Georgopoulos LJ, Kellam P, Blair GE. Identification of genes differentially expressed as result of adenovirus type 5- and adenovirus type 12-transformation. *BMC Genomics* 2009; 10:67.
264. Yang Y, McKerlie C, Lu Z, Wang L, Buchwald M. In vivo potential effects of adenovirus type 5 E1A and E1B on lung carcinogenesis and lymphoproliferative inflammation. *J Virol* 2008; 82:8105-11.